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(54) Title: COMPOSITIONS AND METHODS FOR PRODUCING CELLULAR LABELS FOR NUCLEAR MAGNETIC RESONANCE TECHNIQUES

(57) Abstract: The disclosure provides, in part, compositions and methods for producing cellular labels for tracking cells by MRI. The disclosure provides, in part, methods for labeling, detecting and quantifying cell numbers in vivo.

WO 2009/009105 A2

# COMPOSITIONS AND METHODS FOR PRODUCING CELLULAR LABELS FOR NUCLEAR MAGNETIC RESONANCE TECHNIQUES

## RELATED APPLICATIONS

This application claims the benefit of priority to United States provisional application serial numbers 60/959,135, filed July 10, 2007, and 61/062,710, filed January 28, 2008. The disclosures of each of the foregoing applications are hereby incorporated by reference in their entirety.

## BACKGROUND

Many biological processes are carried out by populations of cells. For example, cells of the immune system are recruited from the bloodstream to areas of inflammation or infection, resulting in an accumulation of immune cells at the affected site. A marked infiltration of immune cells often occurs in tissues affected by autoimmune diseases, cancers and infections. Likewise, transplant rejection is mediated by host immune cells that enter and destroy the transplanted tissue. There is also growing evidence that stem cells originating in the bone marrow migrate through the bloodstream and assist in the regeneration of damaged tissues.

Furthermore, the most immediately promising area of biologic therapy involves the emerging field of cellular therapy. Cellular therapy is broadly defined as the treatment of human disease by the administration of therapeutic cells that have been selected, multiplied, and pharmacologically treated outside the body, or *ex vivo*. These cells may be derived from the patient (autologous cells), from another human (allogenic cells), from other organisms (xenogenic cells), or from immortalized cell lines.

Cells represent the ultimate therapeutic system because of their ability to carry out complex functions and their responsiveness to changes in the surrounding tissue or host organism. In the simplest mode of cellular therapy, cells can be isolated, grown in quantity *ex vivo*, and implanted in patients to produce and secrete soluble factors that directly address the mechanism of disease. Cells can also accomplish tasks as complex as reconstitution of tissues, organs, or immune responses based on their ability to home to specific sites within the body, to exit from circulation, and to integrate into specific tissue

or differentiate into new tissue. Other cellular therapeutics can be programmed for tumor killing or treating metastases (e.g., immunotherapeutics).

Although dynamic cell populations play a key role in significant diseases, present technologies for monitoring the location and movement of cells *in vivo* are quite limited. Typically, cell movements are monitored only in "snap shots" obtained by histological analysis of tissue biopsies. However, the process of sampling a tissue often alters the behavior of cells, and only a limited number of biopsies can be obtained from a particular tissue or organ. Some progress has been made studying cell movements via *in vitro* assays and isolated tissues *ex-vivo*. Existing instruments for non-invasive analysis of living organisms are, at present, ill-suited for tracking living cells. Light-based imaging technologies, such as bioluminescence (e.g. luciferases) technologies, are often ineffective at visualizing deep structures because most mammalian tissues are optically opaque. Positron emission tomography (PET) techniques using radioactively-labeled probes are highly sensitive. However, PET instrumentation is often limited to a resolution of several millimeters and is unable to resolve fine details of tissues and organs. Furthermore, labeled cells cannot be detected for time periods that extend beyond a typical PET radioisotope half-life, and generally PET is not useful for longitudinal studies. In order to gain a fundamental understanding of cellular processes, new ways to visualize and quantify the population dynamics of specific cell types *in vivo* must be developed.

Magnetic resonance imaging (MRI) is a widely used clinical diagnostic tool because it is non-invasive, allows views into optically opaque subjects, and provides contrast among soft tissues at reasonably high spatial resolution. Conventional MRI focuses almost exclusively on visualizing anatomy and has no specificity for any particular cell type. The 'probe' used by conventional MRI is the ubiquitous proton ($^1$H) in mobile water molecules. New classes of exogenous MRI probes or reagents are needed to facilitate cell-specific imaging in living subjects.

## SUMMARY

In certain aspects, the disclosure provides novel methods and reagents for labeling cells *ex vivo* with an imaging reagent, such as fluorocarbon imaging reagent that can be detected by a nuclear magnetic resonance technique. In certain aspects, the disclosure provides methods and software for quantifying the numbers of labeled cells at particular

locations *in vivo*. Cells may be labeled with a label including a fluorocarbon, for example a perfluoropolyether (PFPE), and since biological tissues have negligible endogenous fluorine content, *in vivo* $^{19}$F MRI can provide an effective means of detecting labeled cells. In some embodiments these images are then superimposed on a conventional $^{1}$H MRI to determine anatomical localization.

Labeled cells may be administered to a subject and subsequently detected by nuclear magnetic resonance techniques. Examples of nuclear magnetic resonance (NMR) techniques include MRI and localized magnetic resonance spectroscopy (MRS). Because nuclear magnetic resonance techniques are generally performed as non-invasive procedures, the labeled cells may be detected at one or more time points in a living subject. Labeled cells may also be detected in a cell culture or in essentially any other milieu on which a nuclear magnetic resonance technique can be performed, such as tissue explants, organs and tissues removed from a subject (possibly prior to transplant into a transplant recipient), artificially generated tissues and various matrices and structures seeded with cells.

In certain aspects, the disclosure provides methods for labeling a cell. Such methods may include contacting the cultured cells *ex vivo* with a fluorocarbon imaging reagent under conditions such that the fluorocarbon imaging reagent becomes associated with the cell. Perfluoropolyethers (PFPEs) are examples of suitable fluorocarbon imaging reagents. An imaging reagent may be formulated as an emulsion, often including a surfactant. Optionally, the cell may be contacted with the fluorocarbon imaging reagent in the presence of a reagent that enhances uptake of the fluorocarbon imaging reagent. Various cationic molecules, such as cationic lipids, protamine sulfate and polyethylenimine (PEI), are examples of a suitable uptake enhancing reagent; other such reagents are described herein and are, in view of this specification, known in the art. In certain embodiments, the composition of the surfactant may be designed to impart a cationic surface to the emulsion particle that enhances cellular uptake of the emulsion without the need of an enhancing reagent. In certain embodiments, the uptake enhancing compound is conjugated to the fluorocarbon. In certain embodiments, the cells are labeled with perfluorocarbon emulsion particles by electroporation.

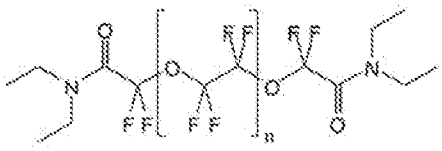
While a fluorocarbon imaging reagent may be internalized by a cell, it may also associate with the extracellular surface of a cell. Association with an extracellular surface

may be increased by conjugating the imaging reagent to a cellular targeting moiety. A cellular targeting moiety may be essentially any molecular entity that binds to the desired cells, such as an antibody that binds to an epitope that is exposed to the extracellular milieu. Uptake of an imaging reagent into a cell may be increased by conjugating the imaging reagent to an internalization moiety. An internalization moiety is any molecular entity that stimulates or promotes entry of the imaging reagent into the cell. Examples include internalizing peptides and moieties that bind to receptors or other cell surface proteins that are internalized by, for example, receptor mediated endocytosis. The cell may be essentially any cell, including prokaryotic and eukaryotic cells. In preferred embodiments, the cell is a mammalian cell. In certain embodiments the cell is a cell of the immune system, such as a dendritic cell or T cell. A cell may also be a stem cell or a cell that has been prepared for administration to a subject as part of a cellular therapy or a transplant, such as a peripheral blood stem cell transplant or bone marrow transplant. Other cell types can be labeled and imaged, for example an embryonic stem cell, a pancreatic islet, a hepatocyte, etc., perhaps in conjunction with a therapy.

In certain aspects, the disclosure provides methods of labeling cells with novel fluorocarbon imaging reagents. Preferred fluorocarbon imaging reagents have one or more of the following properties: reduced cytotoxicity; a $^{19}F$ NMR spectrum that is simple, ideally having mostly a single, narrow resonance to minimize chemical shift artifacts; a large number of NMR-equivalent fluorine atoms per molecule; and suitability for formulation to permit efficient labeling of many cell types.

The present invention provides novel perfluoropolyether compounds (e.g., compounds of formulae 1-41), including purified preparations of those compounds, that may be used as imaging reagents in methods of the invention. The present invention also provides novel compositions comprising defined mixtures of the novel perfluoropolyether diamide compounds of the invention.

For example, in certain aspects, the disclosure provides a compound of any one of formulae 1-9:



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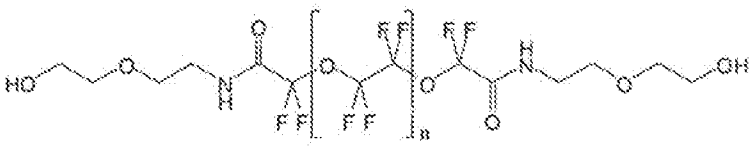

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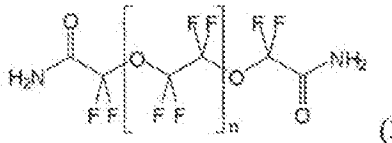

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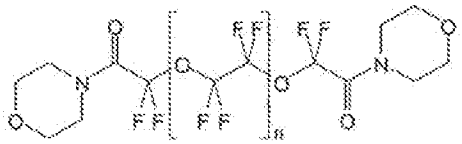

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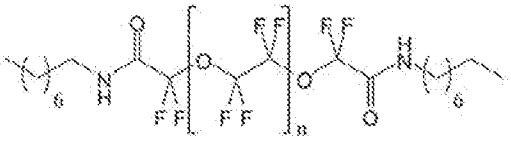

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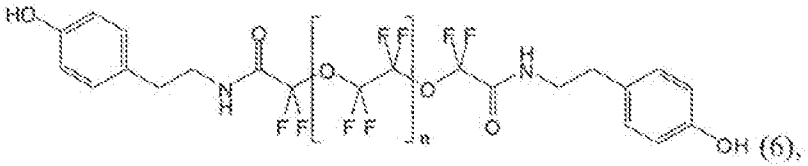

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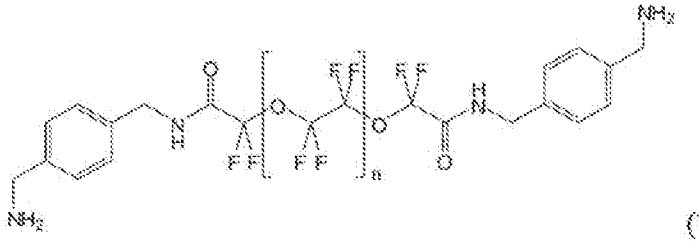

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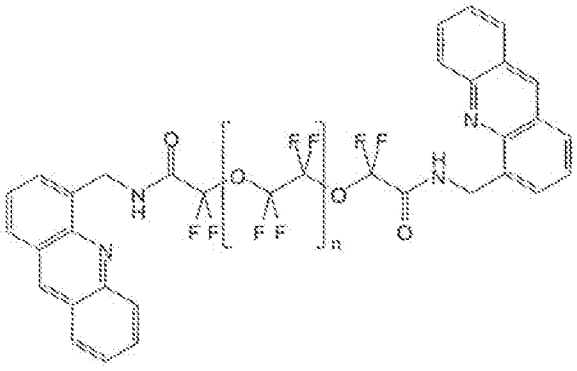

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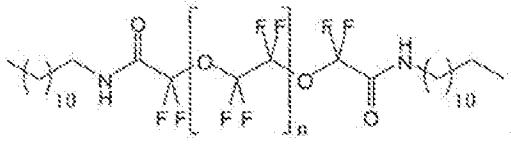
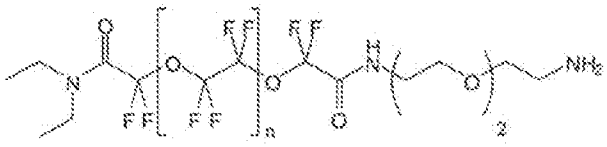


(9),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of any one of formulae 10-15: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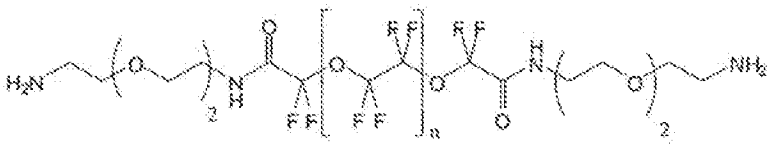
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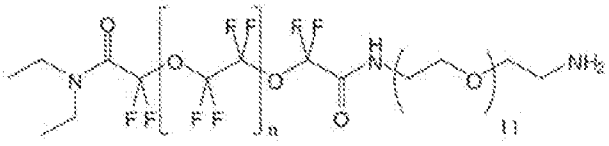
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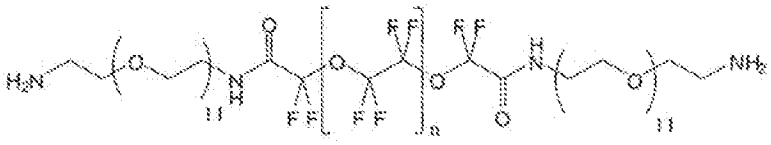
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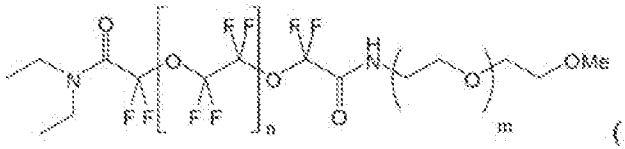
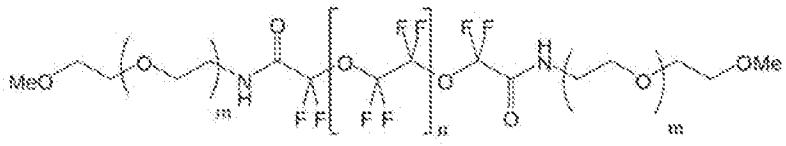

(14), or

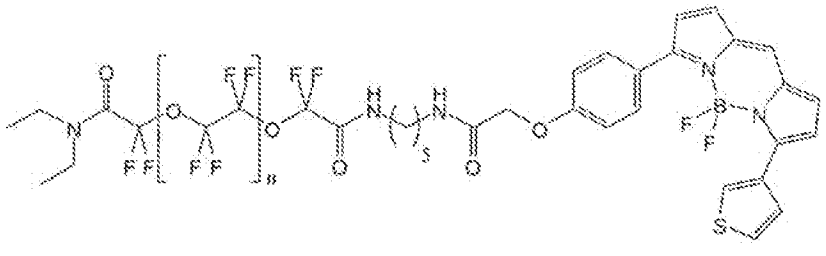

(15),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

m, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of any one of formulae 16-17, or 40-41: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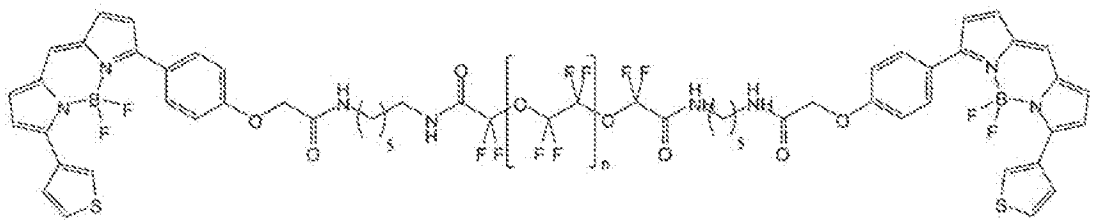
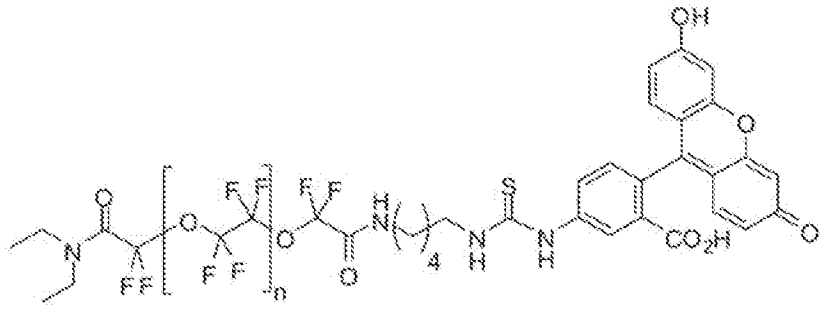
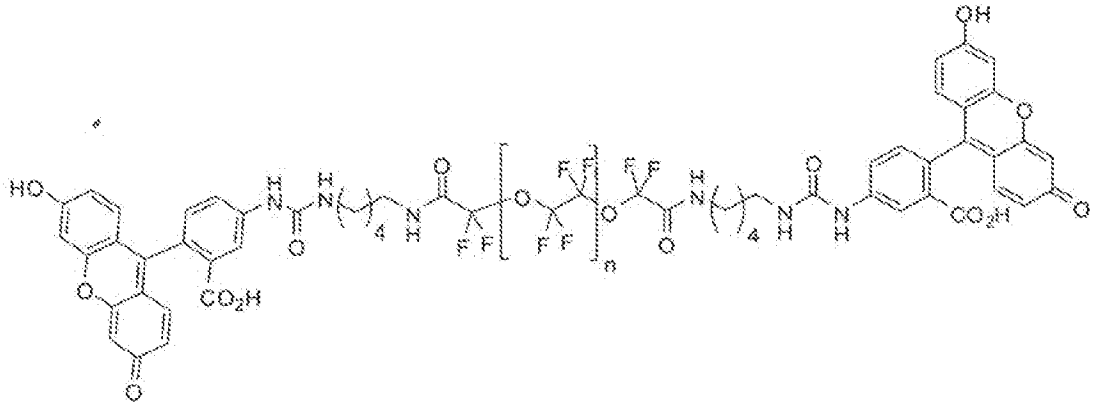


(17),



(40), or

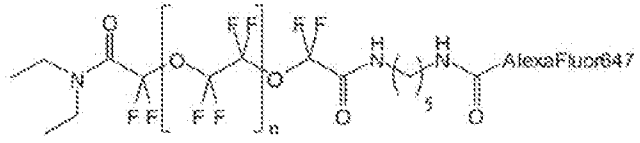


(41),

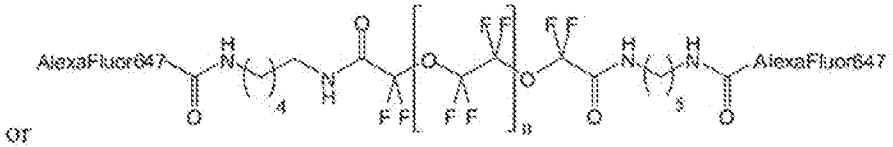
wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of any one of formulae 18 or 19:



(18),

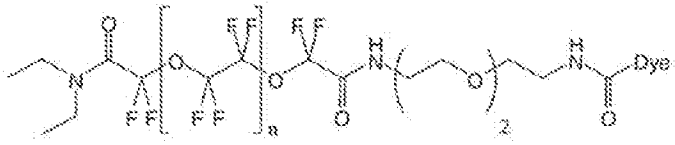


or (19),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 26:



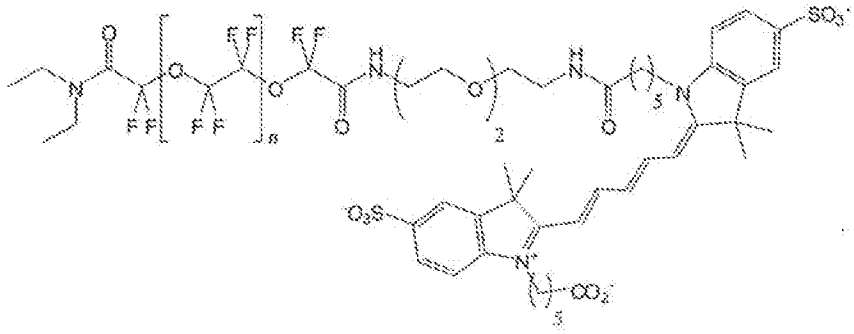
(26),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such embodiments, the compound of formula 26 is a compound of formula 20:

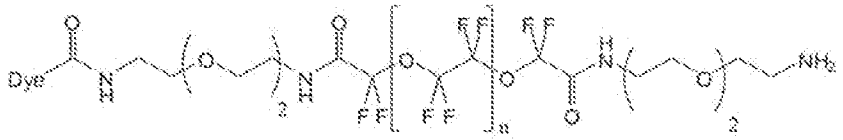


(20),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 27:



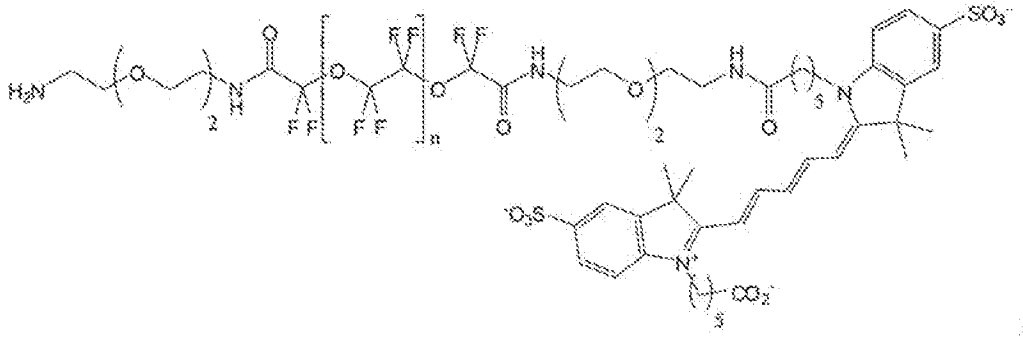
(27),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such aspects, the compound of formula 27 is a compound of formula 21:

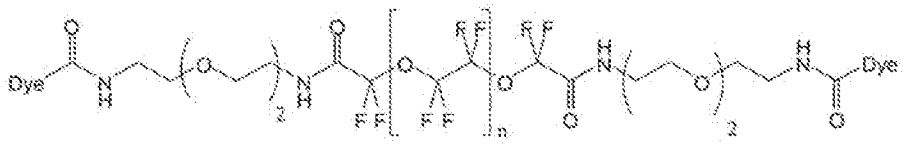


(21),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 28:



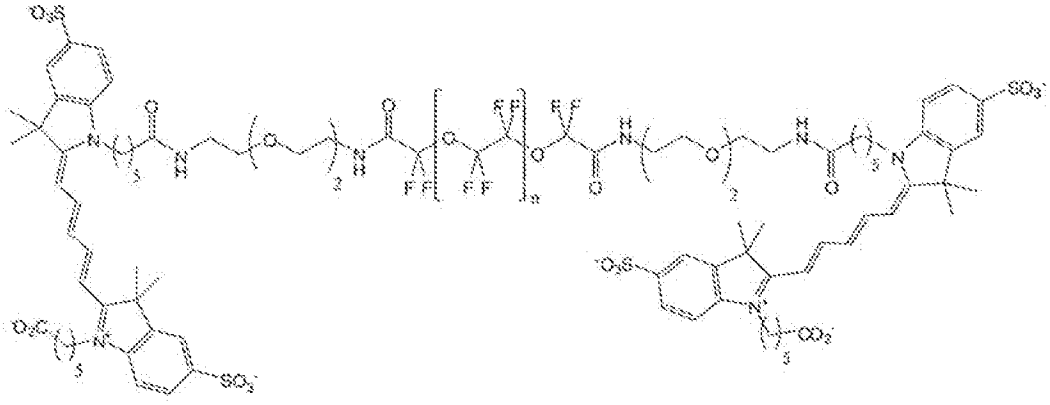
(28),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such aspects, the compound of formula 28 is a compound of formula 22:

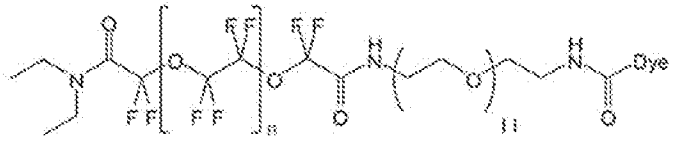


(22),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 29:



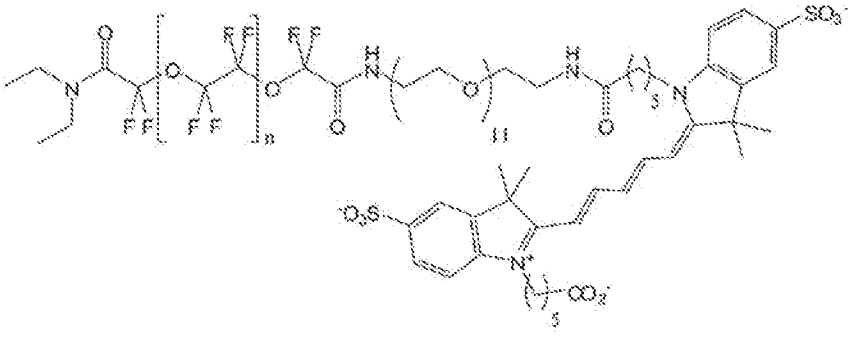
(29),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such aspects, the compound of formula 29 is a compound of formula 23:

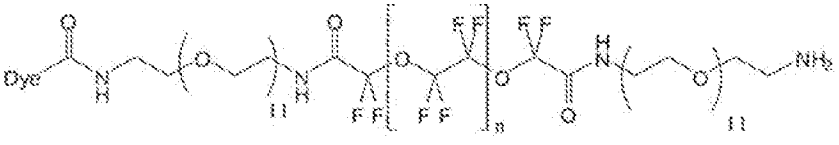


(23),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 30:



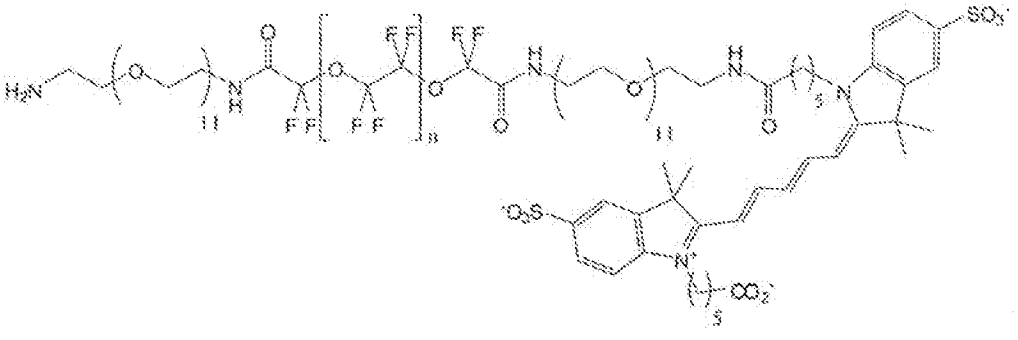
(30),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such aspects, the compound of formula 30 is a compound of formula 24:

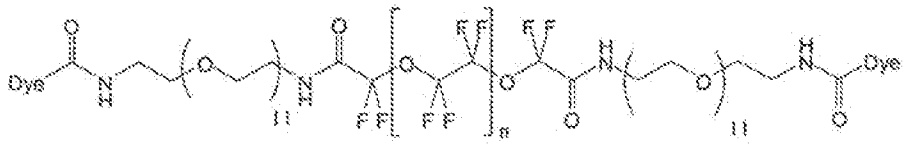


(24),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain aspects, the disclosure provides a compound of formula 31:



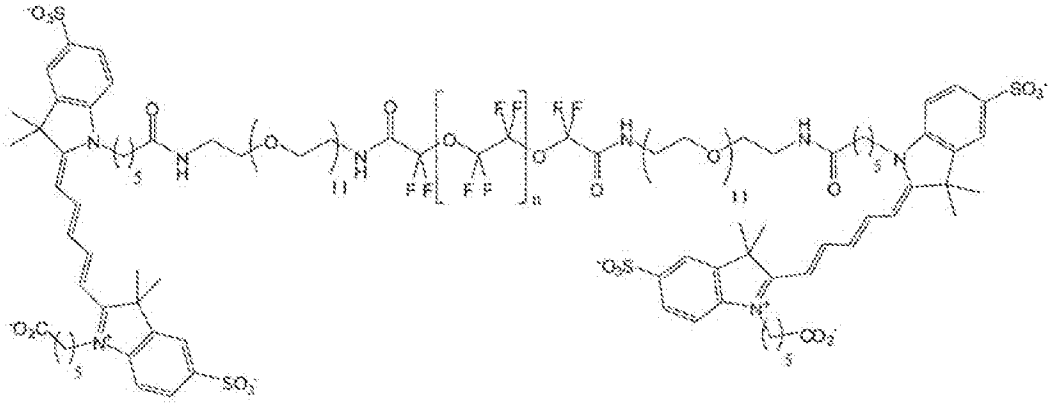
(31),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain such aspects, the compound of formula 31 is a compound of formula 25:

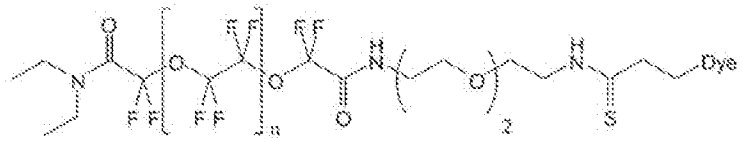


(25),

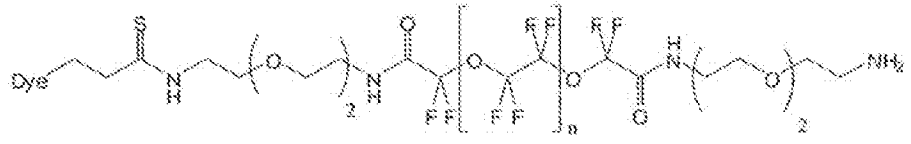
wherein

n, independently for each occurrence, represents an integer from 4 to 16.

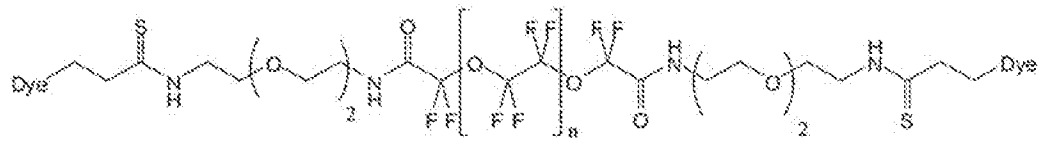
In certain aspects, the disclosure provides a compound of any one of formulae 32-37:



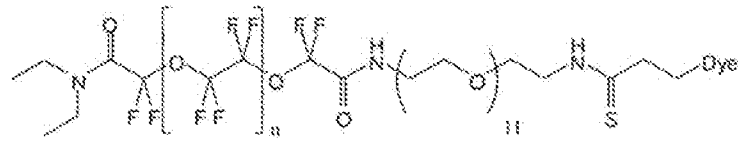
(32),



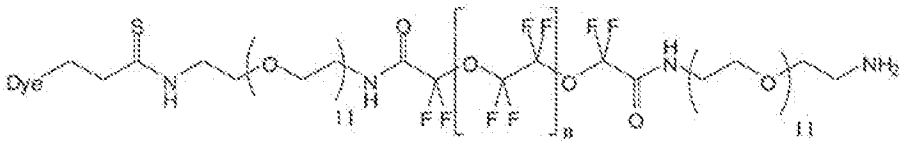
(33),



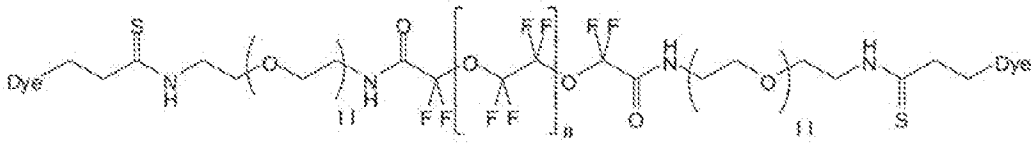
(34),



(35),



(36), or



(37),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye, independently for each occurrence, represents a fluorescent detection moiety.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10 and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12 and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 14 and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 16 and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 40 and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 16 and a compound of formula 17. In certain such embodiments, the composition further comprises a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 19 and a compound of formula 19. In certain such embodiments, the composition further comprises a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 40 and a compound of formula 41. In certain such embodiments, the composition further comprises a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10 and a compound of formula 20.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10, a compound of formula 20, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 21.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 22.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12 and a compound of formula 23.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12, a compound of formula 23, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 24.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 25.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10 and a compound of formula 26.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10, a compound of formula 26, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 27.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 28.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12 and a compound of formula 29.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12, a compound of formula 29, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 30.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 31.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10 and a compound of formula 32.

In certain aspects, the disclosure provides a composition comprising a compound of formula 10, a compound of formula 32, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 33.

In certain aspects, the disclosure provides a composition comprising a compound of formula 11 and a compound of formula 34.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12 and a compound of formula 35.

In certain aspects, the disclosure provides a composition comprising a compound of formula 12, a compound of formula 35, and a compound of formula 1.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 36.

In certain aspects, the disclosure provides a composition comprising a compound of formula 13 and a compound of formula 37.

In certain aspects, the disclosure provides a composition comprising one or more compounds of any one of formulae 1-17 or 20-37 or 40-41 and a compound of formula 1a. In certain embodiments, the composition may comprising one or more compounds of any one of formulae 1-37 or 40-41. In certain embodiments, the composition comprises 80-95% v/v of the compound of formula 1a.

In certain aspects, the disclosure provides a composition comprising one or more compound of any one of formulae 1-17 or 20-37 or 40-41 and perfluoro-15-crown-5 ether. In certain embodiments, the composition may comprising one or more compounds of any one of formulae 1-37 or 40-41. In certain embodiments, the composition comprises 80-95% v/v of perfluoro-15-crown-5 ether.

In certain aspects, the disclosure provides a composition comprising compound of formula:

(42).

In certain aspects, the disclosure provides an emulsion comprising a compound of any one of formulae 1-17 or 20-37, 40-41, 1a, or perfluoro-15-crown-5 ether. In certain aspects, the disclosure provides an emulsion comprising a compound of any one of formulae 1-20-37, 40-41, 1a, or perfluoro-15-crown-5 ether. In certain aspects, the disclosure provides an emulsion comprising a composition comprising a compound of formula 10 and a compound of formula 1; a compound of formula 12 and a compound of

formula 1; a compound of formula 14 and a compound of formula 1; a compound of formula 16 and a compound of formula 1; a compound of formula 10 and a compound of formula 20; a compound of formula 10, a compound of formula 20, and a compound of formula 1; a compound of formula 11 and a compound of formula 21; a compound of formula 11 and a compound of formula 22; a compound of formula 12 and a compound of formula 23; a compound of formula 12, a compound of formula 23, and a compound of formula 1; a compound of formula 13 and a compound of formula 24; a compound of formula 13 and a compound of formula 25; a compound of formula 10 and a compound of formula 26; a compound of formula 10, a compound of formula 26, and a compound of formula 1; a compound of formula 11 and a compound of formula 27; a compound of formula 11 and a compound of formula 28; a compound of formula 12 and a compound of formula 29; a compound of formula 12, a compound of formula 29, and a compound of formula 1; a compound of formula 13 and a compound of formula 30; a compound of formula 13 and a compound of formula 31; a compound of formula 10 and a compound of formula 32; a compound of formula 10, a compound of formula 32, and a compound of formula 1; a compound of formula 11 and a compound of formula 33; a compound of formula 11 and a compound of formula 34; a compound of formula 12 and a compound of formula 35; a compound of formula 12, a compound of formula 35, and a compound of formula 1; a compound of formula 13 and a compound of formula 36; or a compound of formula 13 and a compound of formula 37. In certain aspects, the emulsion further comprises a block copolymer. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38. In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 2900, an average number of PEO units of about 26, such as 26.36, and an average number of PPO units of about 30, such as 30.00. In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO

units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97. In certain aspects, the emulsion further comprises a lipid. In certain such aspects, the lipid is DMPC. In certain aspects, the emulsion further comprising a lipid further comprises a block copolymer. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38. In certain aspects, the emulsion further comprises polyethylenimine. In certain aspects, the emulsion further comprises protamine sulfate. In certain aspects, the emulsion further comprising protamine sulfate further comprises a block copolymer. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38. In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 2900, an average number of PEO units of about 26, such as 26.36, and an average number of PPO units of about 30, such as 30.00. In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97. In certain aspects, the emulsion has a mean particle size of less than 200 nm in diameter. In certain aspects, the emulsion is stable at temperatures ranging from 4 °C to 37 °C. In certain aspects, the emulsion has a polydispersity index ranging from 0.1 to 0.2.

In certain aspects, the disclosure provides an emulsion comprising a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO

units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 16, a compound of formula 17, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-

block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 18, a compound of formula 19, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 40, a compound of formula 41, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-

block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 40, a compound of formula 41, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1a and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5

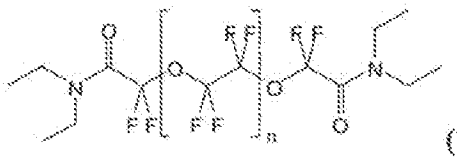
ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number

of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; a compound of formula 1a, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29; perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and protamine sulfate; perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, and an average number of PPO units of about 29, and polyethylamine; perfluoro-15-crown-5 ether and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO

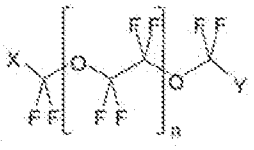
units of about 153, and an average number of PPO units of about 29; a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1a, a compound of formula 1, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1a, a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-

PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate; or a compound of formula 42, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, and an average number of PPO units of about 56, and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate.

In certain aspects, the disclosure provides a method for preparing a composition comprising a compound of formula 1 and a compound of formula 38:



(1) and



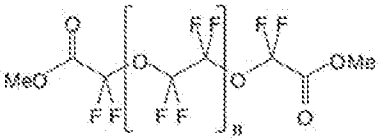
(38),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

one or both of X and Y is an amide other than diethyl amide;

comprising:



1) reacting perfluoropolyether methyl ester (39),

(39), having two methyl ester end groups with a primary or secondary aliphatic amine other than diethyl amine;

2) reacting unmodified methyl ester end groups with excess diethyl amine; and

3) removing unreacted diethyl amine; and

4) optionally removing non volatile unreacted amine by selective extraction in fluorinated solvents or fluoropus phase solid extraction and filtration

Optionally, an imaging reagent may be formulated as an emulsion. Preferred emulsions will be stable at body temperature (37°C for humans) and at a storage temperature, such as 4°C or room temperature (20-25°C). Preferably an emulsion is designed to facilitate uptake of the imaging agent by the subject cells. An emulsion may have an average particle (or droplet) size of less than 500 nm in diameter (meaning that the emulsion may contain particles larger than 500 nm in diameter, but having an arithmetical mean particle diameter falling less than 500 nm, as calculated by methods known in the art). In certain embodiments, the average particle diameter of the emulsion will be less than 400 nm, or less than 300 nm, or less than 200 nm, or less than 100 nm.

In certain aspects, the disclosure provides a method for preparing an emulsion of a PFPE derivative with a block copolymer using low energy methods. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain embodiments, the method for

preparing an emulsion comprising low energy methods may be used to prepare any of the emulsions of the application. In certain embodiments, the low energy method comprises a thin film method.

In certain aspects, the disclosure provides a method for preparing an emulsion of a PFPE derivative with a block copolymer using high energy methods. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain embodiments, the method for preparing an emulsion comprising high energy methods may be used to prepare any of the emulsions of the application. In certain embodiments, the high energy method comprises microfluidization. In certain embodiments, the high energy method comprises homogenization. In certain embodiments, the high energy method comprises sonication.

In certain aspects, the disclosure provides dual fluorescence-19F MRI/MRS reagents formulated as an emulsion. A method may comprise fluorescent detection of labeled cells in vitro and in vivo.

In certain aspects, the disclosure provides methods for detecting a cell in a subject. A method may comprise: administering to the subject a cell that is labeled with a fluorocarbon imaging reagent and examining at least a portion of the subject by a nuclear magnetic resonance technique. Such analysis may include MRI or MRS, which may include collecting data for and generating an image of $^{19}F$ distribution. Imaging may also include collecting data for and generating a conventional anatomical $^{1}H$ image. In a preferred embodiment, $^{19}F$ and $^{1}H$ images are generated and compared, optionally by superposition or overlay. Optionally, labeled cells may be detected using $^{19}F$ MRS. In a preferred embodiment a conventional anatomical $^{1}H$ image is used as a template to guide the positions of one or more localized voxels for $^{19}F$ MRS. NMR data is understood to include both raw and processed data.

In certain aspects, the disclosure provides a method for quantifying cell number *in vivo*. A method may comprise administering to a subject, cells that are labeled with a fluorocarbon imaging reagent; and examining at least a portion of the subject by a nuclear magnetic resonance technique, thereby detecting labeled cells in the subject; and quantifying the number of labeled cells in a region of interest (ROI). In certain embodiments the disclosure provides a method for quantifying labeled cells in a recipient of a transplant that includes labeled cells.

Calibrating the mean "cellular dose" of labeling agent of a particular cell population may be a pre-requisite for *in vivo* quantitative determinations. The *in vivo* equivalent of the cellular dose will be referred to as the number of $^{19}F$ molecules per cell or cell quantity, but is understood to be any measure of the amount of label per cell *in vivo*. In certain embodiments the mean number of $^{19}F$ molecules per cell or cell quantity of a labeled cell population is first measured (i.e., calibrated) *in vitro* prior to administration of cells to the subject or transplantation. In certain embodiments the mean number of $^{19}F$ molecules per cell or cell quantity of a labeled cell population is measured (i.e., calibrated) contemporaneously with examination of labeled cells. In certain embodiments the mean number of $^{19}F$ molecules per cell or cell quantity of a labeled cell population is calibrated after the labeled cells have been examined. In certain embodiments the mean number of $^{19}F$ molecules per cell or cellular dose of a labeled cell population is calibrated in a test population of cells of a particular type, not necessarily destined for a patient, but used to calibrate cellular dose of labeling agent as a consequence of a particular labeling protocol or set of conditions; the value of cellular dose is then used to for future labeling and *in vivo* imaging experiments in the same population type of cells with the same labeling protocol. In certain embodiments the cellular dose or cell quantity of labeling agent is assayed using a variety of quantitative techniques, for example using the integrated area of a $^{19}F$ NMR spectrum of a cell pellet of a known number of labeled cells. Besides $^{19}F$ NMR, many other quantitative methods can be used to assay the cell quantity or cellular dose of the labeling reagent, as described herein. In certain embodiments, the cell quantity or cellular dose can be represented or deduced from prior data. In certain embodiments, the cellular dose or cell quantity may not be directly counted in $F^{19}$ molecules, but the units of the cellular dose of labeling reagent will be representative of this and will be understood to be equivalent.

In certain embodiments, quantifying includes using a calibrated $^{19}F$ signal in the same field of view as the ROI. A calibrated $^{19}F$ signal is a signal that, by virtue of any of the various calibration techniques described herein, or other techniques that will be apparent from this description, is such that one can deduce a relationship between the signal and the representative number of $^{19}F$ molecules or cell quantity in the ROI within the subject. As an example, calibration may be achieved by placing a vial of known quantity of $^{19}F$ molecules in the MRI detection field during imaging of the ROI. This

permits one to calculate the relationship between the signal strength within the ROI and the number of $^{19}F$ molecules. Alternatively, other nuclei can be used in the calibration standard, such as $^{1}H$.

In certain embodiments, the disclosure provides a method of quantifying the numbers of labeled cells *in vivo* within an ROI. For example, following cell administration, and *in vivo* $^{19}F$ MRI/MRS, one can compare the total (e.g. integrated) $^{19}F$ signal intensity in an ROI to a calibrated $^{19}F$ reference. The $^{19}F$ reference may be, for example, a vessel containing a solution with a known concentration of $^{19}F$ nuclei. The vessel would be placed preferably externally or alongside, or optionally inside, the imaged subject or patient prior to data acquisition. In preferred embodiments, the reference is imaged along with the subject in the same image field of view. Optionally, the reference can be imaged in a separate scan, or no external reference can be used.

By computationally manipulating or combining a key set of parameters from the $^{19}F$ MRI/MRS data set, one can calculate the number of labeled cells present in an ROI as described herein. For example, a key set of parameters may include: (i) the cellular dose of labeling agent (i.e., $F_c$) measured *in vitro*; (ii) *in vivo* $^{19}F$ MRI/MRS data set taken in the subject at one or more time points following labeled cell administration; (iii) the voxel volume; (iv) the in-plane voxel area (i.e., area of the image pixel); (v) optionally, the MRI/MRS data set from the $^{19}F$ reference standard; (vi) optionally, the measured Johnson noise of the $^{19}F$ MRI/MRS data in the subject material; (vii) optionally, the measured signal-to-noise ratio (SNR) of one or more voxels of the $^{19}F$ MRI/MRS data set in the subject material; (viii) optionally, the measured SNR of one or more voxels of the $^{19}F$ MRI/MRS data set from the reference standard; (ix) optionally, the $^{19}F$ NMR relaxation times (T1, T2, and T2*) of the subject material; (x) optionally, the $^{19}F$ NMR relaxation times (T1, T2, and T2*) of the reference standard (for example, see *Magnetic Resonance Imaging, Third Edition,* chapter 4, editors D.D. Stark and W.G. Bradley, Mosby, Inc., St. Louis MO 1999). Those skilled in the art can derive other parameters, combinations of the above set, or derivations thereof, particularly from the $^{19}F$ MRI/MRS dataset, that can be used to quantify the number of labeled cells *in situ*. In certain embodiments the above set of key parameters can be used to derive quantitative or statistical measures of the accuracy or confidence of the measured number of labeled cells.

There are many ways to combine the key parameters (i-x, above), any subsets of these, or any of their combinations or approximations, to estimate the effective number of labeled cells seen by $^{19}F$ MRI in the subject material, denoted by $N_c$. For example, one can use an equation of the form

$$N_c = \frac{[F_R]v}{I_R} \frac{1}{F_c} \sum_{i=1}^{N_{ROI}} I_c^{(i)}$$

where: $N_c$ = total number of labeled cells in the ROI; $[F_R]$ = concentration of $^{19}F$ in the calibrated $^{19}F$ reference solution (or gel); v = voxel volume; $I_R$ = mean intensity of the calibrated $^{19}F$ reference taken with the MRI/MRS scan, averaged over one or more voxels; $F_c$ = average $^{19}F$ cellular dose of the labeling agent measured *in vitro*; $N_{ROI}$ = number of voxels in the ROI containing labeled cells; $I_c^{(i)}$ = image intensity of the $i^{th}$ voxel in the ROI containing labeled cells; i = unitless index for voxels in the ROI containing labeled cells.

In certain aspects, the disclosure provides a calculating system for the quantification of $^{19}F$ labeled cells and optionally, a statistical measure of the uncertainty in the measured cell number. In certain embodiments the disclosure provides a computer; a computer readable medium, operatively coupled to the computer, and computer readable medium program codes that can quantify the number of $^{19}F$ labeled cells in a ROI *in vivo*. In certain embodiments the system calculates the number of labeled cells by ratios of the intensity of $^{19}F$ signal and the volume of labeled cells in a ROI compared to a reference. In certain embodiments the system calculates the number of labeled cells according to a formula, an example of which is stated above. In certain embodiments the quantification comprises relating a calibrated NMR signal to a cellular dose.

In certain aspects, the disclosure provides a computer readable medium having computer readable program codes embodied therein for performing *in vivo* quantification of $^{19}F$ labeled cells and optionally, a statistical measure of the uncertainty in the measured cell number. In certain aspects the computer readable medium program codes calculate the number of $^{19}F$ labeled cells in a ROI detected by a magnetic resonance technique. In certain embodiments the system calculates the number of labeled cells by ratios of the intensity of $^{19}F$ signal and the volume of labeled cells in a ROI compared to a reference. In certain embodiments the system calculates the number of labeled cells according to a formula. In certain embodiments the same computer can be used to calculate a statistical

confidence coefficient accompanying the cell number calculation. In certain embodiments the quantification comprises relating a calibrated NMR signal to a cellular dose.

In certain aspects, the disclosure provides a method for labeling a cell, the method comprising contacting the cell *ex vivo* with a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether under conditions such that the fluorocarbon imaging reagent becomes associated with the cell. In certain aspects, the disclosure provides an emulsion comprising a compound of any one of formulae 1-17 or 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether. In certain aspects, the disclosure provides an fluorocarbon imaging reagent comprising a compound of any one of formulae 18 or 19.

In certain aspects, the disclosure provides a method for detecting a cell in a subject, the method comprising:

a) administering to the subject a cell that is labeled with a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether; and

b) examining at least a portion of the subject by a nuclear magnetic resonance technique, thereby detecting a labeled cell in the subject.

In certain aspects, the disclosure provides a method for detecting transplanted cells in a transplant recipient, the method comprising:

a) administering cells for transplant to a transplant recipient, at least a portion of which cells for transplant are labeled with a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether;

b) examining at least a portion of the subject by a nuclear magnetic resonance technique, thereby detecting the labeled cells.

In certain aspects, the disclosure provides a method for quantifying cell number *in vivo*, the method comprising:

a) administering to the subject cells that are labeled with a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether;

b) examining at least a portion of the subject by a nuclear magnetic resonance technique, thereby detecting labeled cells in the subject; and

c) quantifying the number of labeled cells in a region of interest (ROI).

In certain aspects, the disclosure provides a method for labeling a cell, the method comprising contacting the cell *in vivo* with a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether under conditions such that the fluorocarbon imaging reagent becomes associated with the cell.

In certain aspects, the disclosure provides a method for detecting a cell in a subject, the method comprising:

a) administering to the subject a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether; and

b) examining at least a portion of the subject by a nuclear magnetic resonance technique, thereby detecting a labeled cell in the subject.

In certain aspects, the disclosure provides a labeled cellular formulation for administration to a subject, the formulation comprising:

a) a cell; and

b) a fluorocarbon imaging reagent comprising a compound of any one of formulae 1-17, 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether that is associated with the cell.

In certain embodiments of the above methods, the disclosure provides an emulsion comprising a compound of any one of formulae 1-17 or 20-37, 40-42, 1a, or perfluoro-15-crown-5 ether. In certain embodiments of the above methods, the disclosure provides an fluorocarbon imaging reagent comprising a compound of any one of formulae 18 or 19.

As will be apparent from this disclosure, compositions and methods described herein will be useful in a variety of clinical procedures. For example, the disclosure provides methods for detecting donor cells in a recipient, such as a transplant recipient or a recipient of other types of cell-based therapy. Such a method may comprise administering cells for transplant to a transplant recipient, at least a portion of which cells for transplant are labeled with a fluorocarbon imaging reagent; and examining at least a portion of the

subject by a nuclear magnetic resonance technique, thereby detecting the labeled cells. Detection of the labeled cells may be done once or repeatedly and may be performed so as to provide information about the location and trafficking of labeled cells in the transplant recipient. Examples of cell recipients include recipients of bone marrow transplants (or cellular fractions containing hematopoietic stem cells, commonly but not exclusively derived from bone marrow, peripheral blood or cord blood) and other cell or organ transplant recipients. Organ transplant recipients include recipients of donor organs such as liver, heart, lung, kidney, pancreatic tissue, neural tissue or other transplants. Recipients also include recipients of donor cells, which may be derived directly from a donor (in the case of autologous cells, the "donor" is the same individual as the recipient) or subjected to limited or extensive culturing prior to use. Donor cells may be derived from essentially any tissue that serves as a source of useful cells, and may include stem cells (including precursor cells), such as hematopoietic stem cells, hemangioblasts, hepatic stem cells, neural stem cells, muscle stem cells (e.g. satellite cells), cardiomyocyte precursor cells, pancreatic stem cells, vascular endothelial precursor cells, mesenchymal stem cells, bone or cartilage precursor cells, or may include mature cells, such as dendritic cells, immune cells (e.g., T cells, B cells), chondrocytes, osteoblasts, and the like. Cells for administration may be autologous, heterologous or even derived from another organism, such as a pig. Other aspects of the present invention will be apparent from the disclosure below.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1.** PFPE amide 1 emulsified with Pluronic™ F68 by sonication in 1X HBSS buffer. Emulsion was diluted in 1X HBSS and particle size measured by Dynamic Light Scattering (DLS). Data represents average of two independent measurements, (mean ± SD). Critical micelle concentration (CMC) of PFPE amide 1 is 22.2 mg/mL, in presence of 21.6 mol% F68 and 1X HBSS buffer, was estimated from the intersection of the graph of two curves. Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 2.** Effect of neutral lipophilic fluorescent dye on PFPE amide 1 emulsification. The composition containing BODIPy-PFPE amide 16 and PFPE amide 1 emulsified with

L35 in water by sonication. Lipophilic dye does not significantly affect the particle size and polydispersity of PFPE emulsion. Data represents average of three independent measurements (mean ± SD).

**Figure 3.** Fluorescence intensity of BODIPy labeled PFPE amide oil. The composition containing BODIPy-PFPE amide 16 and PFPE amide 1 emulsified in water with L35. Data represents an average fluorescence intensity measured in triplicate (mean ± SD).

**Figure 4.** The composition comprising PEG-PFPE amide 14 and PFPE amide 1 emulsified by sonication in water without additional emulsifiers or surfactants. Particle size was measured by DLS. The data represents an average of two independent measurements mean ± SD.

**Figure 5.** The composition comprising PEG-PFPE amide 14 and PFPE amide 1 emulsified by sonication in 1X HBSS without additional emulsifiers or surfactants. Particle size was measured by DLS. The data represents an average of two independent measurements (mean ± SD).

**Figure 6.** Particle size measurements of the emulsification of the composition comprising PEG-PFPE amide 10 and PFPE amide 1 with different mol% of 10 in water, without additional emulsifiers or surfactants.

**Figure 7.** Particle size measurements of the emulsification of the composition comprising PEG-PFPE amide 10 and PFPE amide 1 with different mol% of 10 in 1X HBSS without additional emulsifiers or surfactants.

**Figure 8.** Effect of PEG-PFPE-amide 10 on PFPE amide 1 emulsification using Pluronic™ L35. Emulsion was prepared by sonication.

**Figure 9.** Particle size and polydispersity index (PDI) were measured for PFPE amide 1 emulsified with a neutral lipid, DMPC, in various ratios using combination of thin-film and sonication methods. Data represents an average of two measurements (mean±SD).

**Figure 10.** Size and volume distribution of PFPE amide 1 emulsified with neutral lipid DMPC in 1X HBSS. The particle size was determined to be 159 nm with PDI = 0.182 following an average of two independent measurements. Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 11.** Comparing particle size and PDI for low and high energy emulsification process for lipid based PFPE amide 1 emulsions. Sonication significantly decreased emulsion droplet size. Data represents an average of two measurements (mean±SD). Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 12.** Characterization of a DDAB/DMPC/PFPE amide 1 emulsion. Sonication decreased emulsion droplet size and PDI. Data represents an average of two measurements (mean±SD). Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 13.** Dynamic light scattering study of the effects of different amounts of cationic lipid (CTAB) on PFPE amide 1 emulsification, alone and in combination with Pluronics™. No significant change in size and PDI with increasing concentration of CTAB.

**Figure 14.** Reversal of zeta potential sign by addition of cationic lipid to PFPE amide 1 emulsion. Data represents an average of two measurements (mean±SD). Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 15.** Size distribution by light scattering intensity distribution of PFPE amide 1/PEI emulsion in water. Presence of PEI did not affect the size or PDI.

**Figure 16.** Particle size and PDI measurements of protamine sulfate / PFPE amide 1 nanoparticles prepared by sonication. Increasing amounts of Protamine Sulfate increased both size and PDI.

**Figure 17.** Protamine Sulfate / PFPE amide 1 zeta potential measurement. When Protamine Sulfate reached 0.5 % w/w, the particle size increased and the zeta potential

changed its sign to positive. Data represents an average of two measurements (mean±SE). Measurements performed on Zetasizer Nano (Malvern, UK).

**Figure 18.** Dynamic light scattering (DLS) measurements of PFPE amide 1/L35 emulsion coated with protamine sulfate for improved cellular delivery. Presence of protamine sulfate slightly increased particle size of coated particles.

**Figure 19.** Particle size and PDI measurements after microfluidization of PFPE amide 1/F68 emulsion in 1X HBSS. Increased number of passes decreased particle size and PDI.

**Figure 20.** Stability of PFPE amide 1/F68 microfluidized emulsion in 1X HBSS evaluated by DLS measurements over time. Data represents an average of two independent measurements (mean±SD).

**Figure 21.** Electron microscopy (EM) measurements, magnification 150X and 70X respectively, showed well defined droplets with average size 200 nm of a PFPE amide 1/F68 microfluidized emulsion.

**Figure 22.** Stability of microfluidized emulsions containing PFPE amide 1/L35 and PFPE amide 1/L64 prepared in water and stored at room temperature. Data represents an average of two independent measurements (mean±SD). Emulsions were stable for 7 weeks as shown by no changes in size and PDI over time.

**Figure 23.** Stability of PFPE amide 1/F68 emulsions microfluidized with/without PEI (low MW) in water. All emulsions were stored at the original concentration and diluted 10 times in water prior to each measurement. Data represents particle size presented as diameter measured by DLS over time after storage at different temperatures.

**Figure 24.** A) DLS comparison of PFPE-oxide/F68 emulsion to PFPE amide 1/F68 after microfluidization. Both compounds emulsify the same way and show identical particle size and PDI. B) Long term stability (250 days) of PFPE-oxide/F68/PEI emulsion at three

different temperatures 4, 25 and 37°C. Data represents the measured Z average (i.e., diameter in nm units) over time.

**Figure 25.** $^{19}F$ NMR of PFPE-oxide/F68/PEI emulsion. The main PFPE-oxide peak at -91.6 ppm has the same chemical shift as in PFPE-amide 1 emulsions. Additional identifying peaks for PFPE oxide 1a are at -58.951 and -93.641 ppm. Spectra were obtained at 470 MHz. TFA ($CF_3COOH$) (0.1 % v/v) is an internal reference at -76.00 ppm.

**Figure 26.** Stability of PFPE emulsions in presence of serum at 37°C in cell culture media. Emulsions were prepared by microfluidization. The data represents DLS measurements in cell culture media, at the cell labeling concentrations and at different time points, measured without further dilution.

**Figure 27.** Measurements of light scattering intensity and particle size as a function of concentration of PFPE-tyramide 6/F68 emulsion prepared by thin film method and heating in water. Data represents average of three independent measurements (mean±SD). The SD is too small to be visible at the presented graph scale.

**Figure 28.** Stability of PFPE-tyramide 6/F68 emulsion in water. The emulsion was stored at room temperature and diluted 10 times in water prior to each measurement. Data represents an average of two measurements (mean±SD).

**Figure 29.** DLS Measurements for PFPE (2-hydroxyl)ethyloxyethyl amide 2/ F68 emulsion prepared by thin film method in water and 1X HBSS at two different temperatures. Emulsion droplets were significantly smaller in water.

**Figure 30.** Cell Titer Glo assay (Promega) versus cell number. This figure shows representative luminescence correlation curve for estimating cell number and viability following PFPE labeling of dendritic cells (DCs). Data represents the average of measurements done in triplicate (mean ±SD).

**Figure 31.** $^{19}F$ NMR spectrum of PFPE amide 1/L35 emulsion labeled DCs. Cells were labeled for 3h, washed, trypsinized and resuspended in 1X PBS. TFA was used as a reference for signal quantification.

**Figures 32A-32B.** Labeling DCs with different PFPE derivatives emulsified with L35 by sonication. A) Cell number estimated from Cell Titer Glo luminescence-cell number correlation curve. Data represents the average of two independent labeling tests (mean±SD). B) Fluorine content estimated from 19F NMR spectra for DC cell suspensions. Data represents the average of two independent measurements (mean±SD).

**Figures 33A-33B.** Labeling DCs with PFPE derivatives emulsified with L35 by sonication and coated with protamine sulfate prior cell labeling. A) Cell number estimated from Cell Titer Glo luminescence-cell number correlation curve. Data represents the average of two independent labeling tests (mean±SD). B) Fluorine content estimated from 19F NMR spectra for DC cell suspensions. Data represents the average of two independent measurements (mean±SD).

**Figures 34A-34B.** PFPE-tyramide/F68 emulsion uptake in dendritic cells. Tyramine conjugated to PFPE promotes cellular uptake and decreases cellular toxicity. A) Cellular viability estimated from Cell Titer Glo luminescence measurements. B) The 19F uptake measurement shows a linear correlation to the emulsion dose. Data represents the average of two independent measurements.

**Figures 35A-35B.** Jurkat cells labeling with PFPE/L35 emulsion with and without protamine sulfate. A) Cell numbers were estimated from Cell Titer Glo luminescence-cell number correlation curve. B) Fluorine content estimated from 19F NMR spectra for Jurkat cell suspensions. A dramatic increase in uptake for the protamine sulfate coated nanoemulsion droplets was observed. Data represents the average of two independent measurements (mean±SD).

**Figure 36.** 19F NMR spectrum of PFPE amide/L35 labeled T cell pellet. TFA was used as a reference.

**Figure 37.** Long term viability of PFPE labeled Jurkat Cells. Labeled cells were cultured in 96-well plates and viability was tested by Cell Titer Glo at each time point. Data represents the average of triplicate experiments (mean±SD).

**Figure 38**. BODIPy-PFPE amide 16 and PFPE amide 1 emulsified with L35 in water by sonication and used for T cell labeling. PFPE-BODIPy/L35 emulsion uptake in T cells was measured by the fluorescent signal of covalently bound BODIPy-TR dye. These quantitative fluorescence measurements demonstrate a dose dependent uptake.

**Figure 39.** Correlation between fluorescence signal and the $^{19}F$ NMR signal from T cells labeled with PFPE-BODIPy/L35 emulsion. Data represents the average of duplicate experiments (mean±SD). Data demonstrates utility of the dual fluorescent-19F label.

**Figures 40A-40D.** Fluorescent images of DCs labeled with BODIPy-PFPE/L35 emulsion (A is untreated, B is labeled) and BODIPy-PFPE/L35 coated with protamine sulfate (C is untreated, D is labeled).

**Figure 41.** Jurkat cells labeled with PFPE-oxide/F68 emulsions with and without PEI. Uptake was increased by PEI several fold. The data represents an average of triplicate experiments (mean±SD).

**Figure 42.** Dose dependent uptake of PFPE-oxide/F68/PEI emulsion in non-phagocytic cells (Jurkat cells) labeled in suspension. Data represents the average of triplicate experiments (mean±SD).

**Figure 43.** Alexa647-PFPE amide/L35 emulsion measurements, where A) is the absorbance standard curve, B) is the fluorescence standard curve, and C) shows that the emulsion fluorescent emission spectrum is the same as that of the free dye.

**Figure 44.** GC/FID traces of PFPE amide and the PFPE emulsion product. Both samples show the same polymer distribution and retention times under the conditions employed.

**Figure 45.** $^{1}$H NMR of PFPE amide 1 and starting material PFPE ester 39. The disappearance of methyl group peak (4.1ppm) serves as an indicator for the PFPE ester to PFPE amide conversion and was used for monitoring coupling reactions.

**Figure 46.** $^{13}$C NMR of PFPE amide 1 in $d_3$-trifluoroethanol solvent that completely solubilizes PFPE.

**Figure 47.** $^{13}$C NMR of PFPE ester 39.

**Figure 48.** $^{19}$F NMR spectrum of PFPE amide 1. The diagnostic peak for the preterminal CF2 group is at -74.4 ppm, where the TFA reference is set to -76 ppm.

**Figure 49.** $^{19}$F NMR spectrum of PFPE ester. The diagnostic peak for the preterminal CF2 group is at -80.3 ppm. The chemical shift of this peak was used as an indicator for amine coupling and amide synthesis.

**Figure 50.** MALDI-TOF mass spectrometry analysis of PFPE amide 1.

**Figure 51.** $^{1}$H NMR of PFPE-tyramide purified on FluoroFlash by selective fluorophobic solvent elution.

**Figure 52.** $^{19}$F NMR of PFPE-tyramide. The diagnostic peak is at -78.8 ppm for the preterminal CF2 group.

**Figure 53.** A functional block diagram of a general purpose computer system 200 for performing the functions of the computer 104 according to an illustrative embodiment of the invention.

**Figure 54.** A) Long term stability (250 days) at three different temperatures of PFPE-oxide/F68 and B) PFPE-oxide/F68/protamine sulfate nanoemulsion prepared by microfluidization. Measurements were made by DLS. The nanoemulsion with protamine

sulfate shows greater stability at higher temperatures (25 and 37°C). At low temperature (4°C) both emulsions remained stable for 250 days.

**Figure 55.** Uptake dose curves of PFPE-oxide/F68 emulsions with/without protamine sulfate in DCs. Data represents the average of two independent measurements (mean±SD). A dramatic increase in uptake (e.g., almost one order of magnitude) was observed for the PFPE oxide 1a nanoemulsion with incorporated protamine sulfate.

**Figure 56.** Activated primary T cells labeled with BODIPy-PFPE amide 16 and PFPE amide 1 blended with PFPE oxide 1a and emulsified with F68 and PEI in water by microfluidization. A) DIC image; B) cell surface stained with CD4-FITC (green) antibody; C) BODIPy-PFPE nanoemulsion (red).

**Figure 57.** Correlation between the $^{19}F$ NMR and fluorescence in non-phagocytic Jurkat cells labeled with blended BODIPy-TR PFPE amide/PFPE oxide 1a nanoemulsion following 3h co-incubation. Data represents the average of duplicate experiments (mean±SD). Data demonstrates utility of the dual fluorescent-19F label.

**Figure 58.** Fluorescent images of DCs labeled with blended nanoemulsion. The BODIPy-TR PFPE blended nanoemulsion is shown where A) shows a DIC image and B) shows BODIPy-TR PFPE (red). The FITC PFPE blended nanoemulsion is also shown where C) is the DIC image and D) shows FITC PFPE (green).

**Figure 59.** BODIPy-TR Cadaverine concentration versus absorbance calibration curve. The absorbance was measured at a fixed absorption wavelength 593 nm, which was experimentally determined as the absorbance maxima in trifluoroethanol. Data represents the average of three independent measurements (mean ± SD).

**Figure 60.** 9L cells incubated with FITC PFPE amide/Perfluoro-15-crown-5 nanoemulsion prepared with F68 and Protamine Sulfate by microfluidization. The cells were exposed to increasing concentrations of the nanoemulsion for 3h. A) Cell numbers were estimated from Cell Titer Glo luminescence-cell number correlation curve. B)

Fluorine content estimated from 19F NMR spectra for 9L cell suspensions. Data represents the average of two independent measurements (mean±SD).

**Figure 61.** RAW cells incubated with BODIPy-TR PFPE amide/Perfluoro-15-crown-5 nanoemulsion prepared with P105/Cremophor EL mixed micelle. The cells were exposed to increasing concentrations of the nanoemulsion for 24h and cell loading was measured by $^{19}$F NMR. The data (cell loading, $^{19}$F/cell) represents an average of duplicate measurements (mean±SD).

**Figure 62.** 19F NMR spectrum of RAW 264.1 cells labeled with BODIPy-TR PFPE amide 1/Perfluoro-15-crown ether/P105/Cremophor EL nanoemulsion. Cells were incubated with the nanoemulsion for 24h, washed, scrapped and resuspended in 1X PBS. TFA (-76.00 ppm) was used as a reference for signal quantification. Labeled cells are shown at -92.47 ppm in the spectrum.

**Figure 63.** Effect surfactant amount on $CF_3$ nanoemulsion uptake in DCs. A) Uptake in DCs measured by 19F NMR; B) Cell viability upon 3h exposure to different nanoemulsions. Data represents the average of two independent measurements (mean ± SD).

**Figure 64.** Dose dependent uptake of nanoemulsion A4 in DCs upon 3h incubation. Uptake was measured by $^{19}$F NMR. Data represents the average of 4 independent measurements (mean ± SD).

**Figure 65.** $^{19}$F NMR spectrum of nanoemulsion A4 labeled DCs. Labeled cells are represented with the peak at -72.5 ppm, while TFA serves as a reference at -76.0 ppm.

DETAILED DESCRIPTION

1. Overview

In certain aspects, the disclosure provides novel methods and reagents for labeling cells *ex vivo* with a nuclear magnetic resonance imaging reagent, such as a fluorocarbon imaging reagent and quantifying the labeled cells *in vivo* or *ex vivo*. In certain aspects, the disclosure provides novel methods and reagents for labeling cells *in vivo* with a nuclear magnetic resonance imaging reagent. Labeled cells may be detected by a $^{19}F$ nuclear magnetic resonance technique (e.g., MRI/MRS) and quantified according to methods described herein. $^{19}F$ nuclear magnetic resonance techniques are excellent imaging tools for biological systems because of the absence of endogenous background signals. Fluorine is present, if at all, at exceedingly low levels in living organisms, and generally not in a chemical form that is detectable by liquid-state nuclear magnetic resonance techniques. This is quite distinct from conventional $^{1}H$ MRI which, while providing visualization of fine anatomical detail, does not permit selective detection of particular cell populations. Certain methods disclosed herein permit whole or partial body screening to visualize the distribution of labeled cells in a living subject. The precise anatomical location of labeled cells detected by $^{19}F$ nuclear magnetic resonance may be determined by, for example, superimposition of a $^{1}H$ MRI image that provides anatomical detail. In preferred embodiments, the $^{1}H$ image is acquired during the same imaging session as the $^{19}F$ image (without moving the subject) to ensure registration. Additionally, the nuclear magnetic resonance techniques disclosed herein may be applied effectively in *ex vivo* contexts, as in the case of tissue samples, excised organs and cell cultures. The imaging technology disclosed herein may be applied to a large number of biological and medical problems.

In certain aspects, a method of the invention may comprise labeling cells *ex vivo* with a $^{19}F$ imaging reagent, administering the labeled cells to a subject, and detecting labeled cells in the subject. The cells to be labeled may be a crude cellular fraction or tissue sample, or the cells may be cultured and/or subjected to enrichment prior to labeling. For example, particular cell types may be selected by fluorescence activated cell sorting (FACS) prior to labeling. Other sorting or selective enrichment methods are known in the art for the various different cell types that may be of interest. The types of cells that are labeled may also be controlled by the nature of the imaging reagent. For example, simple colloidal suspensions of imaging reagent will tend to be taken up more quickly by cells

with phagocytic activity. As another example, an imaging reagent may be formulated with or covalently bound to a targeting moiety that facilitates selective targeting of the imaging reagent to a particular population of cells. Imaging reagents are described further below. After labeling, cells may be immediately administered or the cells may be stored, further cultured, purified, enriched, segregated or processed in any way that is not incompatible with the intended use of such cells.

In certain aspects, labeled cells will be administered for a therapeutic purpose. Technology described herein may be used for monitoring the trafficking of cellular therapeutics *in vivo* or in any other desired milieu, such as a tissue explant. Bone marrow cell transplants have been widely used for many years in recipients of ablative therapies for cancers. Various purified cell populations have also been used in place of bone marrow, such as cell populations enriched for hematopoietic stem cells; for example cells may be harvested from umbilical cord blood or peripheral blood. After entering the bloodstream, the stem cells generally travel to the bone marrow, where they begin to produce new white blood cells, red blood cells, and platelets. This engraftment usually occurs within about 2 to 4 weeks after transplantation. Traditionally, engraftment is monitored by testing blood counts on a frequent basis, and complete recovery of immune function generally requires several months (for autologous transplant recipients) to years (for patients receiving allogeneic or syngeneic transplants). Cell sampling by bone marrow aspiration can provide further information on the function of the transplanted cells. These monitoring techniques may be enhanced by *ex vivo* labeling of the cells to be transplanted (or some small fraction of such cells), thus permitting non-invasive monitoring of the location and movement of transplanted cells by nuclear magnetic resonance techniques. Non-myeloablative allogeneic transplantation (i.e. reduced-intensity transplant) is a similar cell therapy that can be effective for treating several types of cancer. Generally, this technique relies on a lower dose of radiation and/or chemotherapeutic and a limited graft-versus-host disease (the action of immune cells from the transplant against any residual host cancer cells) to provide sufficient anti-cancer activity, as well as the hematopoietic potential of the graft cells to restore the patient's hematopoietic system. As with a traditional ablative graft, the techniques of the present invention may be used to monitor the locations and movements of graft cells in a non-myeloablative allogeneic transplantation.

Cellular therapeutics are also in development for use in the delivery of therapeutic proteins. In one embodiment, cells can be isolated, grown in quantity *ex vivo* and then implanted to produce and secrete soluble factors, which may be active either locally (e.g. enzymes, cytokines, and neurotransmitters) or at a distance (e.g. hormones and growth regulators). Cells may also be administered to a patient in order to accomplish complex therapeutic purposes, such as reconstitution of tissues, organs, or immune responses based on their ability to home to specific sites within the body, exit from the circulation, and integrate into surrounding tissue or differentiate to replace damaged tissue. Stem cell therapies have also been proposed for myriad diseases including neurological disorders, particularly those characterized by cell death (e.g., Parkinson's disease, stroke and brain injury caused by trauma), cardiovascular disorders (e.g., myocardial infarction), muscle regeneration (e.g., in patients suffering from cachexia or other wasting disorders), pancreatic regeneration in diabetes, liver regeneration, etc. In each instance, cells, or a sub-population thereof, may be labeled with an imaging reagent *ex vivo* prior to administration, thus allowing the monitoring of these cells *in vivo*. *In vivo* monitoring by a nuclear magnetic resonance technique may be useful, for example, to evaluate the viability of the administered cells. A doctor may tailor a dosing schedule depending on the degree to which labeled cells are detected in a patient after administration. *In vivo* monitoring may also be useful in determining whether therapeutic cells have localized to a desired location. In general, it will be possible to investigate correlations between the migration behavior of therapeutic cells *in vivo*, as well as the number and/or survivorship of therapeutic cells *in vivo*, and therapeutic outcomes. When such correlations have been established, the *in vivo* imaging of therapeutic cells may be used as a prognostic indicator that may be helpful in selecting the appropriate dosage, administration modes and additional therapeutic interventions that will benefit the patient. Certain imaging advances of the invention will benefit a broad range of cellular therapeutic strategies because these imaging methodologies will be able to detect *when*, *where* and *if* the therapeutic cells have been delivered to the desired targets *in vivo*. Additionally, the detection of labeled cells may be enhanced by quantification of labeled cells in a ROI, such as a particular organ or tissue.

One example of an application of technology disclosed herein is in tracking dendritic cells (DCs). DCs are known to be the most efficient antigen presenting cells and

have the capacity to stimulate naive T cells to initiate an immune response. Because DCs are the most potent stimulators of immune response in the body, DCs represent a possible therapeutic approach to increasing the "visibility" of tumors to a patient's immune system. DCs are the focus of tumor vaccines in development. Varying methods are used to expose the dendritic cells to tumor antigens *ex vivo*, after which educated dendritic cells are reinfused to stimulate development of T-cell mediated tumor killing. Data applying an embodiment of the present disclosure to the labeling and tracking of DCs and other cell types, presented in WO2005072780, is incorporated by reference herein.

In addition to DCs, other cell types have demonstrated promise for immunotherapy in cancer and other diseases such as diabetes, although their progress has been hampered by many factors, including the inability to observe their movement following transplantation into animals and humans. Natural killer (NK) cells, when harvested, treated *ex vivo*, and transplanted, have demonstrated the ability to kill metastatic tumor cells. Additional cell types treated *ex vivo* and transplanted to promote cancer immunity include lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes, and activated killer monocytes. Transplantation of T cells, which are white blood cells that attack pathogenic cells, has demonstrated promise against a variety of cancers, including pancreatic cancer, in which clinical trials are beginning, and against multiple sclerosis and HIV infection.

In certain aspects, labeled cells are administered to a subject for non-therapeutic purposes. For example, cells may be labeled *ex vivo*, administered to a subject and then detected, with the expectation that the labeled cells will behave similarly to like, unlabeled cells *in vivo* and may therefore be used to monitor the behavior of endogenous cell populations. Monitoring may be used for the purpose of tracking movements of cells, particularly in the case of cells that are known to be highly mobile, such as cells of the immune system, many types of stem cells and blood born cells. Monitoring may also be used for the purpose of tracking viability or adherence of non-mobile cells at the site of implant. Cells of many tissues, such as muscle, liver, pancreas, kidney, brain or skin will tend to be relatively stationary, but disappearance of label may indicate a high death rate, low adherence, or other information. Modern cell culture and sorting techniques allow the selective pooling and labeling of virtually any desired cell population, including various stem cell types, immune cell types, and other blood cell types. For example, cell surface

markers can be used to sort mixed populations of cells to purify a population of interest. As described in WO2005072780 and US provisional application No. 60/792003 (both of which are herein incorporated by reference in their entirety), both T cells and dendritic cells may be labeled *ex vivo* and detected *in vivo*.

As an example, labeled immune cells may be used as detectable proxies for the movements of immune cells in a patient. Immune cells participate in and are markers for a host of inflammatory and autoimmune disorders, as well as cancer and atherosclerotic plaque formation. As a general methodology, any process involving the recruitment of immune cells may be detected in a patient by administering to the patient labeled immune cells. The accumulation of label in a particular area provides an indication of the degree of immune response occurring in that portion of the body. Traditionally, these types of studies involve histological techniques that are incompatible with living subjects. Certain methods of the disclosure may facilitate the development of therapeutic strategies for the treatment of human diseases. The ability to track selected populations of immune cells non-invasively, and without the use of radioisotopes, can impact many areas of basic and clinical immunology, such as multiple sclerosis, diabetes, monitoring organ transplant rejection, and cancer. For instance, tumors are often highly infiltrated by immune cells. Labeled cells may be imaged in a subject to reveal the location of a tumor, and in some instances may be useful as a non-invasive detection screen. Early detection of cancers has been a critical problem, as most early stage cancers are readily treated by surgery without resort to debilitating chemotherapeutic agents. Likewise, the progress of other inflammatory diseases may be monitored by tracking the dynamics of immune cells in the patient. The effectiveness of immunosuppressant therapy may be assessed as well. In the instance of an organ transplant recipient, the recipient could receive a dose of labeled immune cells prior to receiving the transplantation. *In vivo* monitoring of the accumulation of immune cells in the transplant could then be used as an early warning sign of rejection. In the case of transplants, the methods disclosed herein are particularly desirable because the alternative, biopsies, are well-known to increase the risk of organ rejection.

As an additional example, cells for use in a bone marrow cell transplant, or a peripheral blood stem cell transplant, may be labeled *ex vivo* as described herein, administered, and monitored *in vivo* by a nuclear magnetic resonance technique. Such

monitoring may be used to evaluate the engraftment of donor cells in the recipient bone cavities, as well as survivorship and movement of labeled cells in the recipient. A physician can use information relating to the trafficking of donor cells in a recipient as an early indication of the likely success or failure of the procedure. This type of early detection will allow physicians to tailor the post-transplant therapeutic regimen accordingly. Another cellular cancer therapeutic where the detection technology can be applied is the allogeneic non-myeloablative, or reduced intensity transplant. This procedure may be used with a donor lymphocyte infusion to boost graft-versus-tumor effect which destroys cancer cells. Here the entire population, or a fraction, of transplanted cells could be labeled before infusion. A nuclear magnetic resonance technique could then be used determine where the cells traffic to in the body, which can be indicative of the efficacy of the procedure. As it is often desirable to limit the dose of allogeneic cells to minimize rejection, the cell's trafficking pattern may be used to calibrate dose. In the above cancer cell therapies it may be desirable to selectively label one or more sub-population of the transplanted cells (e.g., CD34+ stem cells or T cells) that are believed to have therapeutic efficacy.

As a further example, cells involved in formation of new tissue, such as in angiogenesis, can be labeled, administered to a subject, and detected to identify hotspots of tissue formation. For example, smooth muscle cells and/or endothelial precursor cells may be labeled and introduced into the bloodstream. Such cells are expected to accumulate at sites of angiogenic activity. Angiogenic activity may be associated with physiological and pathological events such as menstrual cycling, early pregnancy, collateral vessel formation in response to arterial blockages, tumor development and wound healing. Similarly, cells involved in wound healing, such as fibroblasts, may be labeled and administered systemically or to a site of suspected injury in order to monitor cellular behavior.

For example, a medicament or delivery device containing labeled cardiomyocyte lineage cell aggregates or cells derived therefrom may be provided for treatment of a human or animal body, including formulations for cardiac therapy. Cardiomyocyte lineage cells may be administered to a patient in a method for reconstituting or supplementing contractile and/or pacemaking activity in cardiac tissue (see US Patent Application No. 20060040389, 20050112104, 20050244384, which are incorporated in their entirety herein).

In accordance with the present invention labeled cardiomyocyte lineage cells are used to regenerate or repair striated cardiac muscle that has been damaged through disease or degeneration. The labeled cardiomyocyte lineage cells integrate with the healthy tissue of the recipient to replace the function of the dead or damaged cells, thereby regenerating the cardiac muscle as a whole. Cardiac muscle does not normally have reparative potential. The labeled cardiomyocyte lineage cells are used, for example, in cardiac muscle regeneration for a number of principal indications: (i) ischemic heart implantations, (ii) therapy for congestive heart failure patients, (iii) prevention of further disease for patients undergoing coronary artery bypass graft, (iv) conductive tissue regeneration, (v) vessel smooth muscle regeneration and (vi) valve regeneration.

The administration of the cells can be directed to the heart, by a variety of procedures. Localized administration is preferred. The mesenchymal stem cells can be from a spectrum of sources including, in order of preference: autologous, allogeneic, or xenogeneic. There are several embodiments to this aspect, including the following. The present invention allows monitoring of the progress of these cell *in vivo*.

The cardiomyocyte lineage cells may be cardiomyocyte precursor cells, or differentiated cardiomyocytes. Differentiated cardiomyocytes include one or more of primary cardiomyocytes, nodal (pacemaker) cardiomyocytes; conduction cardiomyocytes; and working (contractile) cardiomyocytes, which may be of atrial or ventricular type. In certain embodiments, cells come from a muscle sample (or other sample) that contains muscle progenitor cells such as satellite cells (see US Patent Application No. 20050244384). In certain embodiments, cells are mesenchymal stem cells (MSCs) (see US Patent Application No. 20050112104).

A "cardiomyocyte precursor" is defined as a cell that is capable (without dedifferentiation or reprogramming) of giving rise to progeny that include cardiomyocytes. Such precursors may express markers typical of the lineage, including, without limitation, cardiac troponin I (cTnI), cardiac troponin T (cTnT), sarcomeric myosin heavy chain (MHC), GATA4, Nkx2.5, N-cadherin, .beta.1-adrenoceptor (.beta.1-AR), ANF, the MEF-2 family of transcription factors, creatine kinase MB (CK-MB), myoglobin, or atrial natriuretic factor (ANF).

In certain instances, cells may prove to be so thoroughly associated with a biological site or structure of interest that the labeled cells may be administered for the sole

purpose of aiding in the visualization of such a structure. As mentioned above, immune cells characteristically infiltrate tumors. Accordingly, labeled immune cells may be administered for the purpose of visualizing tumors.

Technology disclosed herein may be applied to studies of animal models of human diseases. Various animal models of diseases may evince altered dynamics or survival of one or more cell populations. Such cell populations may be labeled, administered to the animal and monitored. For example, the infiltration of immune cells into the pancreas of the NOD mouse model for diabetes may be monitored. Other examples of animal models include: experimental allergic encephalomyelitis (multiple sclerosis model), gliosarcoma tumor models, and organ-transplant rejection. By tracking phenotypically-defined populations of immune cells in these models, one can elucidate aspects of the disease etiology and monitor how cell trafficking is affected by therapeutics. This method may be used, for example, to screen for drugs that have a desired effect in an animal model. A drug screening assay may comprise administering labeled cells to an animal and detecting the cells *in vivo* in the presence of a test agent. Changes in cell behavior that are correlated with the presence of the test agent may be indicative of a therapeutic effect. Such changes may be detected by comparison to a suitable reference, including, for example, the same animal before and after treatment with the test agent or a separate, untreated animal. In addition to a test agent, the methods may be used to evaluate the effects of test conditions, such as an exercise regimen, injury, genetic alteration, etc. As an example, it is expected that a drug for treatment of an autoimmune disease would decrease the tendency of immune cells to accumulate in an affected tissue. In addition to steady state evaluations, methods disclosed herein may be used to evaluate kinetic properties of cells, such as the rate at which cells arrive at a particular site and the time of signal persistence at a site. Drug screening assays may be particularly powerful when combined with *in vivo* monitoring of tightly defined cell populations, such as certain groups of immune cells that are implicated in various disorders. For example, monitoring of labeled cytotoxic T cells may be particularly useful in identifying drugs that may be useful in preventing transplant rejection. The ability to monitor cells *in vivo* provides a powerful assay that may be applied to the analysis of essentially any experimental animal, including, for example, any of the various transgenic or otherwise mutant mice that have been generated.

Several groups have studied labeling and visualizing immune cells using MRI contrast agents. Other researchers have used MRI contrast agents to label cell types such as stem cells and neuronal precursors. The majority of these studies render the cells magnetically-distinct via the incorporation of superparamagnetic iron-oxide (SPIO) agents. Cells labeled with contrast agents incorporating other types of metal ions, particularly gadolinium and manganese have also been used. In studies utilizing these metal-ion based agents, the compounds are not directly imaged; instead, one observes their indirect effect on surrounding waters. The presence of the agent tends to shorten the relaxation times ($T_1$, $T_2$, or $T_2^*$) of water in proximity to the compound; these effects can be detected in relaxation time-weighted images. SPIO agents, for example, impart contrast to conventional $^1H$ images by locally perturbing the magnetic field experienced by the nearby mobile water molecules, which in turn modulates $T_1$, $T_2$, or $T_2^*$. Methods described herein are distinctly different from all methods using metal ion based contrast agents because signals from $^{19}F$ nuclei in the imaging reagents may be directly detected and, optionally, imaged.

An inherent drawback to detecting labeled cells using metal-ion based contrast agents is that one is often in a situation where it is necessary to interpret subtle changes in grayscale contrast in regions that are believed to contain labeled cells. The large $^1H$ background signal from the high concentration of mobile water present in tissues can make it difficult to unambiguously identify regions containing labeled cells; this is especially problematic if the labeled cell biodistribution is not known *a priori*. The results of a 'snapshot' image are often ambiguous as to whether labeled cells are present in a specific tissue. This is a particularly vexing problem when trying to detect SPIO labeled cells in iron-laden organs that intrinsically appear dark in anatomical ($T_2$-or $T_2^*$-weighted) images, such as in the liver or the spleen. Often one must resort to detecting the time-lapse image intensity changes in a particular organ over a period of several hours to verify that labeled cells have accumulated. Furthermore, quantification of labeled cells *in vivo* in regions of interest using metal-ion based contrast agents is problematic, and there is generally no simple and reliable way to do this using relaxation-time weighted MRI or by using quantitative relaxation-time MRI maps.

In certain aspects, the disclosure provides multispectral 19F MRI reagents. In certain embodiments, a wide spectral separation between MRI reagents is useful for two

channel MRI imaging, because it allows imaging of one cell type (e.g. DCs labeled at one frequency) simultaneously with another cell type (e.g. T cells, labeled with PFPE). In certain embodiments, this allows for studies of cell-cell interaction *in vivo* by 19F MRI. In certain embodiments, two-channel MRI is to be able to simultaneously track more then one cell type by non-invasive methods and study *in vivo* in real time cell-cell interactions. Such applications are highly relevant, for example, in the fields of cancer and inflammation.

Thus the methods and compositions disclosed herein provide much needed tools in the fields of medicine and biology.

2. Imaging Reagents and Formulations

The imaging reagent used in the subject methods is a fluorocarbon, i.e., a molecule including at least one carbon-fluorine bond. By virtue of the $^{19}F$ atoms, the imaging reagents disclosed herein may be detected by $^{19}F$ MRI and other nuclear magnetic resonance techniques, such as MRS techniques. In certain preferred embodiments, a fluorocarbon imaging reagent will have one or more of the following properties: 1) reduced cytotoxicity; 2) a $^{19}F$ NMR spectrum that is simple, ideally having a single, narrow resonance to minimize chemical shift artifacts; 3) high sensitivity with a large number of NMR-equivalent fluorine atoms in each molecule; 4) formulated to permit efficient labeling of many cell types and not restricted to phagocytic cells. Preferably, the imaging reagent comprises a plurality of fluorines bound to carbon, e.g., greater than 5, greater than 10, greater than 15 or greater than 20 fluorines bound to carbon. Preferably, at least 4, at least 8, at least 12 or at least 16 of the fluorines have a roughly equivalent NMR chemical shift.

For labeling cells in culture, the imaging reagents can be employed in one or more of at least three modalities: 1) imaging reagents that are internalized or otherwise absorbed by target cells without the formation of any covalent or other binding association; 2) imaging reagents that covalently attach to target cells; and 3) imaging reagents coupled to molecules, such as antibodies or ligands, that bind to molecules present on the target cells.

Imaging reagents of the first type include the perfluoro crown ethers and other perfluoropolyethers (PFPEs) that are taken up by cells and, preferably, are retained in the cell without degradation for a substantial period of time, e.g., having a half-life in the cell

of at least 1 hour, at least 4 hours, at least about a day, at least about three days, or even at least about a week. For obvious reasons, it is preferred that the imaging reagent not interfere with ordinary cellular functions or exhibit cytotoxicity at the concentrations employed for labeling. As demonstrated herein, perfluoropolyethers show reduced toxic effect on the labeled cells.

Imaging reagents of the second type include electrophilic compounds that react with nucleophilic sites on the cell surface, such as exposed thiol, amino, and/or hydroxyl groups. Accordingly, imaging reagents such as maleimides, alkyl iodides, N-hydroxysuccinimide or N-hydroxysulfosuccinimide esters (NHS or sulfo-NHS esters), acyl succinimides, and the like can form covalent bonds with cell surfaces. Other techniques used in protein coupling can be adapted for coupling imaging reagents to cell surface proteins. See Means et al. (1990) Bioconjugate Chemistry 1:2-12, for additional approaches to such coupling.

Imaging reagents of the third type can be prepared by reacting imaging reagents of the second type not with the cells themselves, but with a functional moiety that is a cell-targeting ligand or antibody. Suitable ligands and antibodies can be selected for the application of interest. For example, a ligand that selectively targets hematopoietic cells could be labeled with an imaging reagent as described herein and administered to a patient, such as by injection.

Alternatively, an imaging reagent can be coupled to an indiscriminate internalizing peptide, such as antepennepedia protein, HIV transactivating (TAT) protein, mastoparan, melittin, bombolittin, delta hemolysin, pardaxin, Pseudomonas exotoxin A, clathrin, Diphtheria toxin, C9 complement protein, or a fragment of any of these. Cells treated with this indiscriminate molecule *ex vivo* will absorb the imaging reagent. When such labeled cells are implanted into an animal, such as a mammal, the imaging reagent can be used to visualize and/or track the implanted cells by nuclear magnetic resonance techniques.

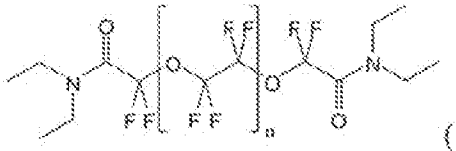
In one embodiment, the internalizing peptide is derived from the drosophila antepennepedia protein, or homologs thereof. The 60-amino acid-long homeodomain of the homeo-protein antepennepedia has been demonstrated to translocate through biological membranes and can facilitate the translocation of heterologous polypeptides to which it is coupled. See for example Derossi et al. (1994) *J Biol Chem* 269:10444-10450; and Perez et al. (1992) *J Cell Sci* 102:717-722. It has been demonstrated that fragments as small as

16 amino acids long of this protein are sufficient to drive internalization. See Derossi et al. (1996) *J Biol Chem* 271:18188-18193.

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy et al. (1989) *Nucl. Acids Res.* 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo, (1989) *Cell* 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell *in vitro* (Green and Loewenstein, (1989) *Cell* 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben et al., (1989) *J. Virol.* 63:1-8). Peptides or analogs that include a sequence present in the highly basic region can be conjugated to fluorinated imaging reagents to aid in internalization and targeting those reagents to the intracellular milieu.

Another PFPE composition of interest is linear PFPEs derivatized with a variety of end groups. The linear compounds have the advantage that one can conjugate a variety of functional entities to the end groups, such as functional moieties of various types. The $^{19}F$ NMR spectra of these linear compounds generally is more complex than the macrocyclic compounds, but a PFPE with two well-separated NMR signals can also be used. In this case it may be desirable to use an MRI pulse sequence that incorporates one or more off-resonance saturation pulses applied to the smaller resonance to eliminate any chemical shift artifacts.

The present invention provides certain novel perfluoropolyether diamide compounds, including purified preparations of those compounds that may be used as imaging reagents in methods of the invention. For instance, the invention provides compounds of any one of Formulae 1-9:



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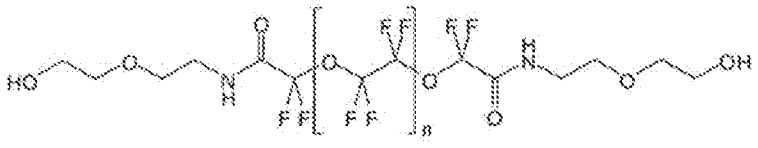

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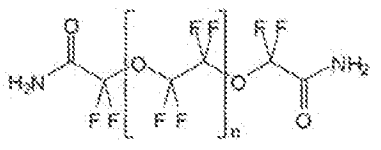

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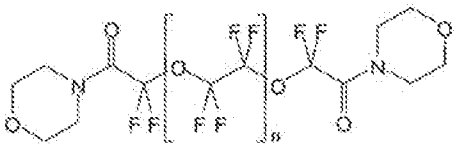

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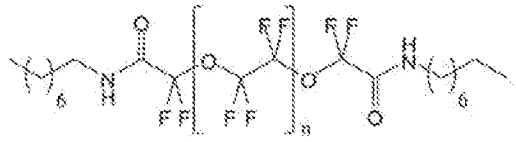

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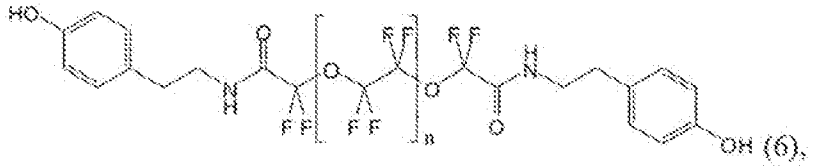

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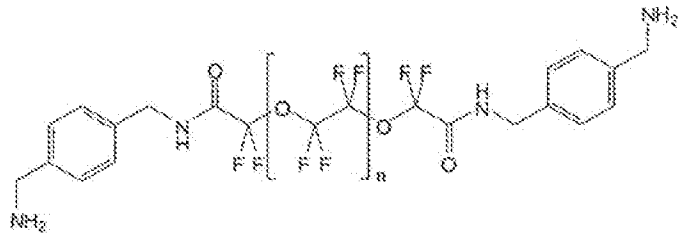

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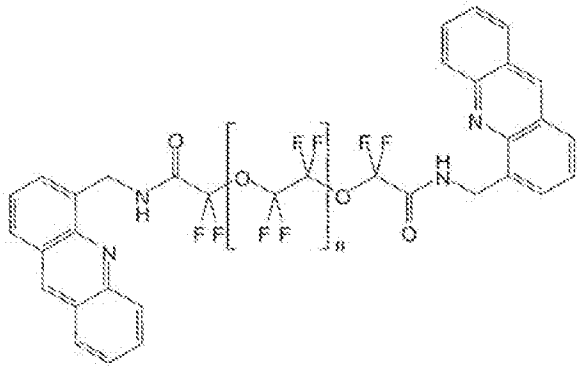
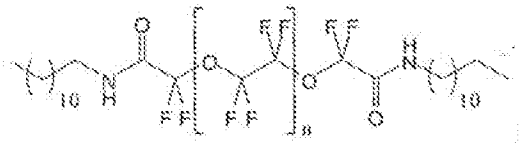


(8), or



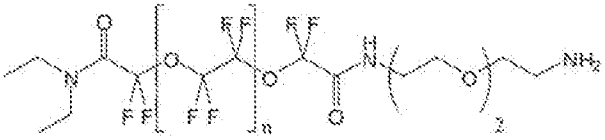
(9),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, a linear perfluoropolyether may be derivatized with a relatively hydrophilic moiety at one or both ends. For example, the hydrophilic moiety may be a polyethylene glycol, thus forming a block copolymer with water-soluble regions on one or both end(s) and a hydrophobic region in the center. When mixed in an aqueous environment, imaging reagents of this type will tend to form micelles, with the PFPE core

surrounded by a water-soluble coat. Amino-PEG blocks are commercially available with a range of molecular weights. In certain embodiments, the invention provides novel perfluoropolyether diamide compounds that contain one or more polyethylene glycol (PEG) groups. These derivatized perfluoropolyether diamide compounds may be used as imaging reagents in methods of the invention. For example, the invention provides compounds of any one of formulae 10-15:



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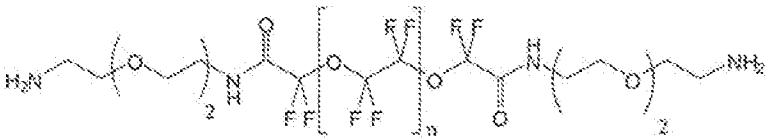

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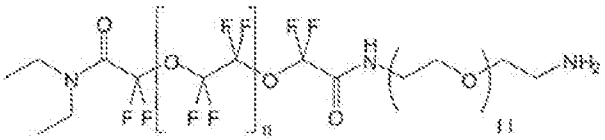

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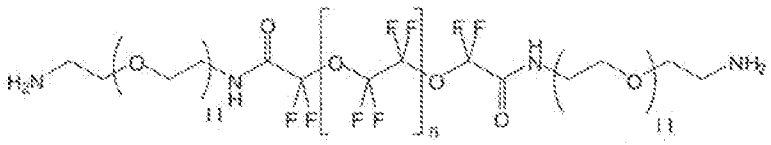

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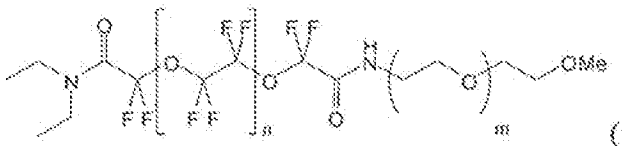
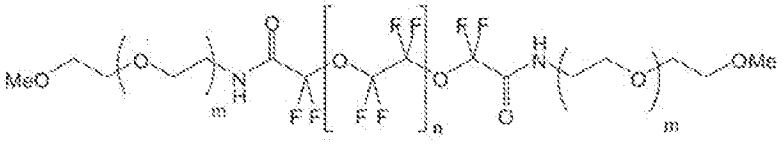


(14), or



(15),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

m, independently for each occurrence, represents an integer from 4 to 16.

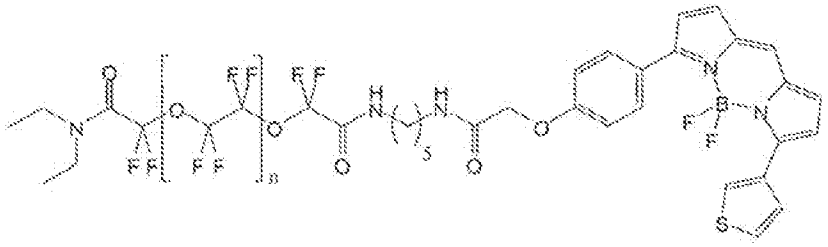
A particularly useful application of linear PFPEs is the synthesis of a "dual mode" agent that can be detected by $^{19}F$ nuclear magnetic resonance techniques and includes a detection moiety that facilitates detection by a second detection method. As an example, a fluorescent moiety attached to the end groups may be used to generate imaging reagents

that can be visualized with $^{19}F$ MRI and fluorescence microscopy. A wide range of fluorescent moieties may be used in a dual-mode agent. Many suitable fluorophores are known, including fluorescein and its derivatives (e.g., Oregon Green 488 and 514, Dichlorofluorescein, Carboxyfluorescein, etc., where all are available from Molecular Probes), lissamine, phycoerythrin, rhodamine (Perkin Elmer Cetus), Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, Alexa dyes (Molecular Probes), BODIPy dyes (Molecular Probes) and FluorX (Amersham). Fluorescent moieties include derivatives of fluorescein, benzoxadioazole, coumarin, eosin, Lucifer Yellow, pyridyloxazole and rhodamine. These and many other exemplary fluorescent moieties may be found in the Handbook of Fluorescent Probes and Research Chemicals (2000, Molecular Probes, Inc.). Additional fluorescent moieties include fluorescent nanocrystals, such as the "quantum dot" products available from Quantum Dot Corporation (Hayward, Calif.). Such nanocrystals may be constructed with a semiconductor core having an appropriate emission spectrum (e.g., CdS, CdSe, CdTe), a shell composed of a non-emissive transparent and relatively non-reactive material that can be efficiently wed to the underlying core material (e.g., ZnS), and a coating that provides desirable solubility (e.g., for solubility in aqueous, physiological solutions) and possible reactive groups for attachment to a fluorocarbon described herein.

Dual mode imaging reagents that permit fluorescent detection are particularly useful in a variety of applications. For example, fluorescent labeling permits the use of fluorescence-based cell sorting mechanisms, such as Fluorescence Activated Cell Sorting (FACS). Cell sorting may be desirable, for example, to enrich for a population of cells that have been successfully labeled. This may be particularly useful where labeling has been directed to rarer cell populations. Dual mode agents are also useful for finding and characterizing labeled cells after they have been implanted into a living subject. In this application, cells may be biopsied, or by some other means harvested, from the subject after they have resided there for some duration. Biological analysis of the harvested cells can then be performed. For example, FACS analysis can be performed on the harvested cells, where after positively selecting cells for the fluorescent PFPE label, the cells can be assayed for the expression of specific cell surface markers (using a different color fluorescent probe) to investigate any change in cell phenotype that occurred following implantation. Fluorescent labels may also be used for fluorescence microscopy of cells,

particularly using three-dimensional confocal fluorescence microscopy. Fluorescence microscopy will not generally be useful for *in vivo* visualization of deep tissues containing labeled cells, but surface tissues may be visualized as well as tissue samples. Dual labeling will be particularly valuable in calibrating and validating any new fluorocarbon-based nuclear magnetic resonance labeling method. Results obtained by, for example, MRI/MRS may be compared to those obtained by fluorescence detection, both in cultured labeled cells (biopsied or otherwise) and *in vivo*, to the extent possible. A known fluorescence signal strength per unit molecule or nanoparticle may be used to calibrate the cellular dose *in vitro* (i.e., Fc).

In certain embodiments, the invention provides novel perfluoropolyether diamide compounds that contain one or more fluorescent detection groups. These derivatized perfluoropolyether diamide compounds may be used as imaging reagents in methods of the invention. For example, the invention provides compounds of any one of formulae 16-19 or 40-41:



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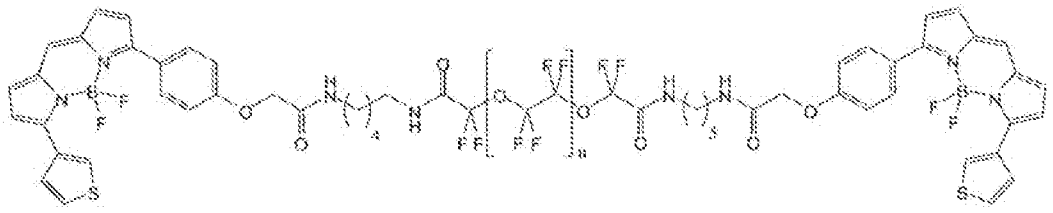

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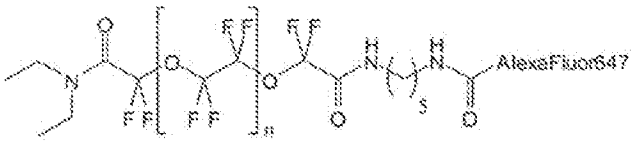


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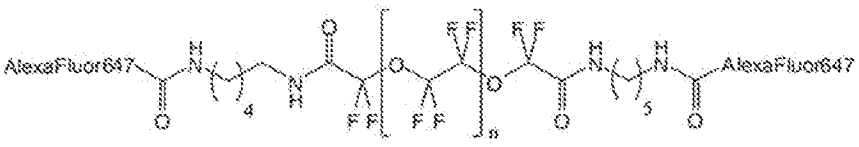


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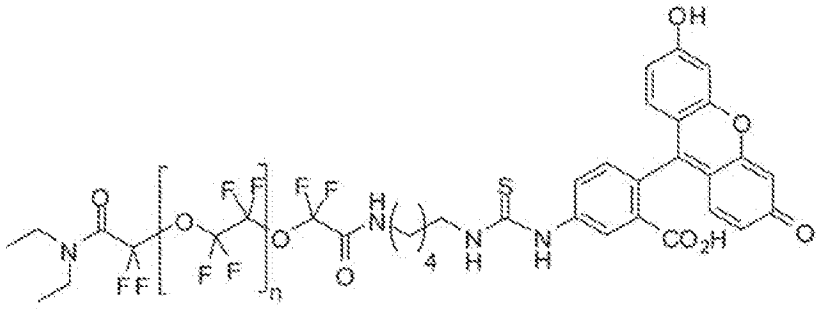

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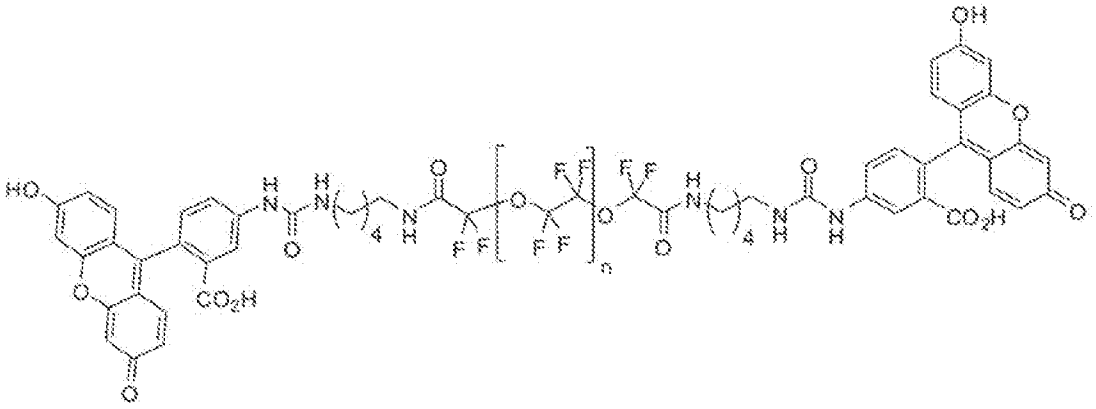


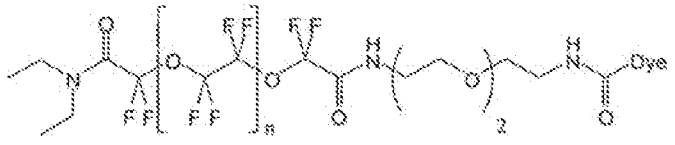
(41)

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

AlexaFluor647 is a residue of AlexaFluor647 fluorescent dye available from Molecular Probes™.

In certain embodiments, the invention provides novel perfluoropolyether diamide compounds that contain one or more fluorescent detection groups and one or more PEG groups. These derivatized perfluoropolyether diamide compounds may be used as imaging reagents in methods of the invention. For example, the invention provides compounds of any one of formulae 26-37:



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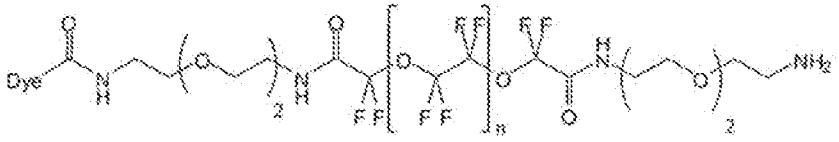

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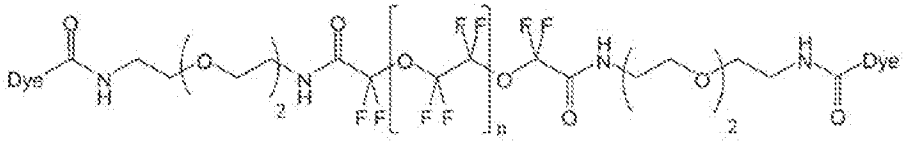
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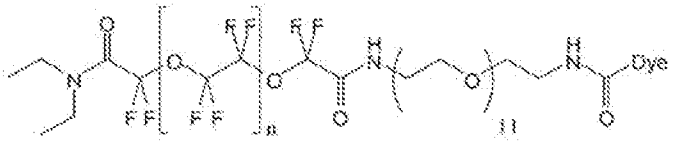
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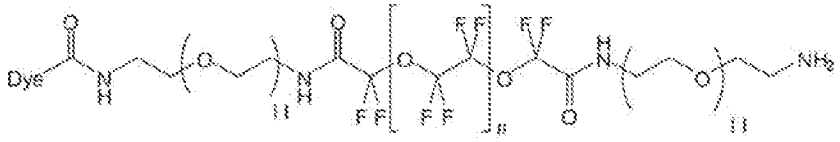
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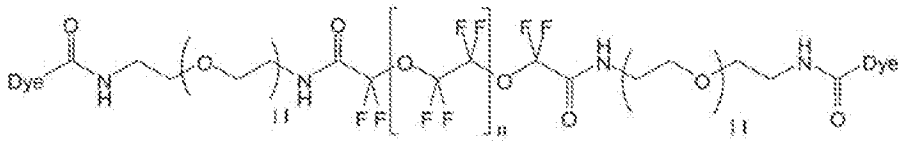
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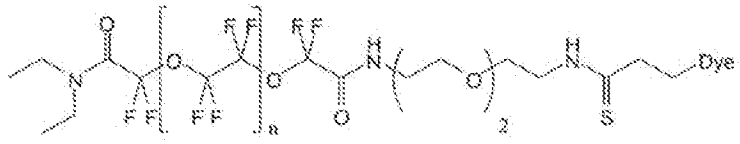
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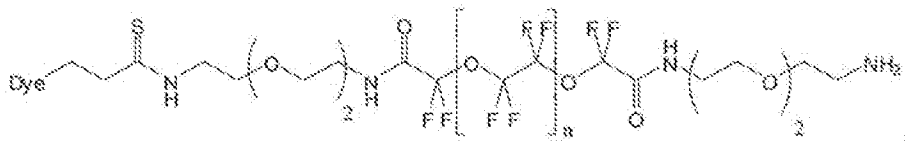
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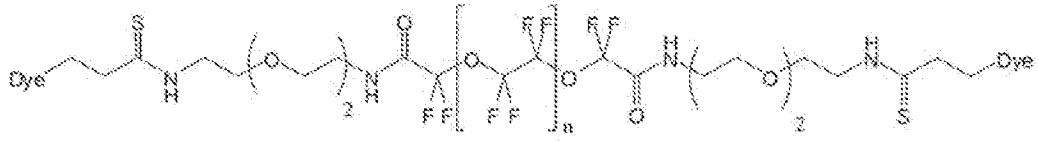
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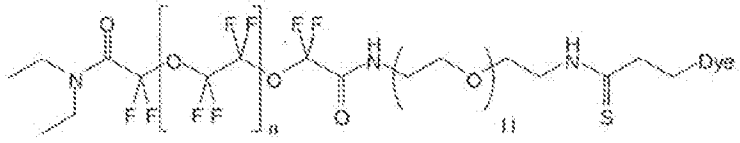
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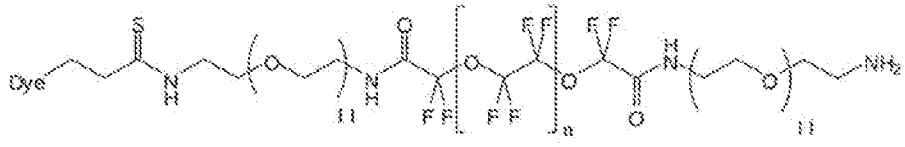
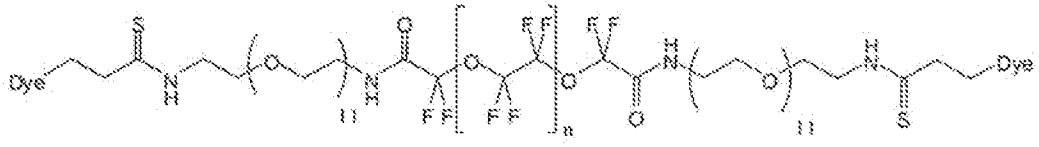

(36), or

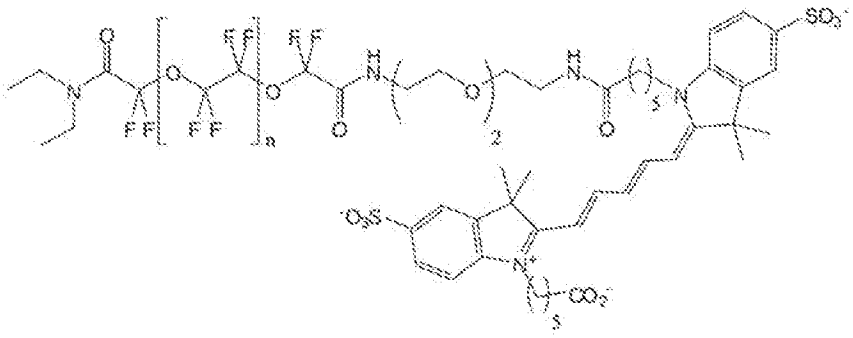

(37),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

Dye represents a fluorescent detection moiety.

In certain embodiments, the compound of formula 26 is represented by a compound of formula 20:

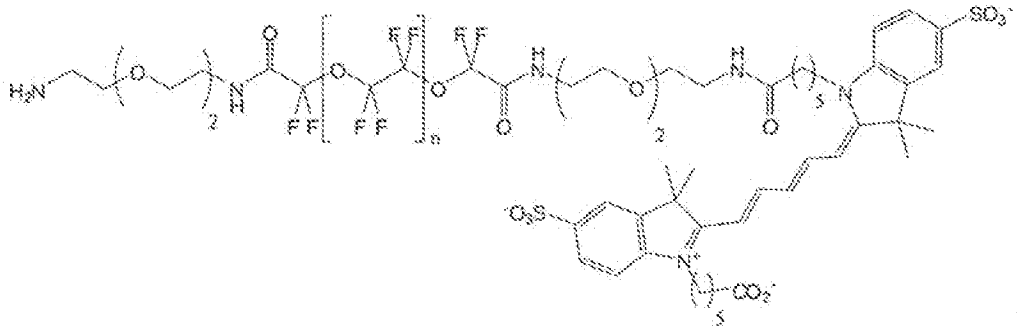


(20),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the compound of formula 27 is represented by a compound of formula 21:

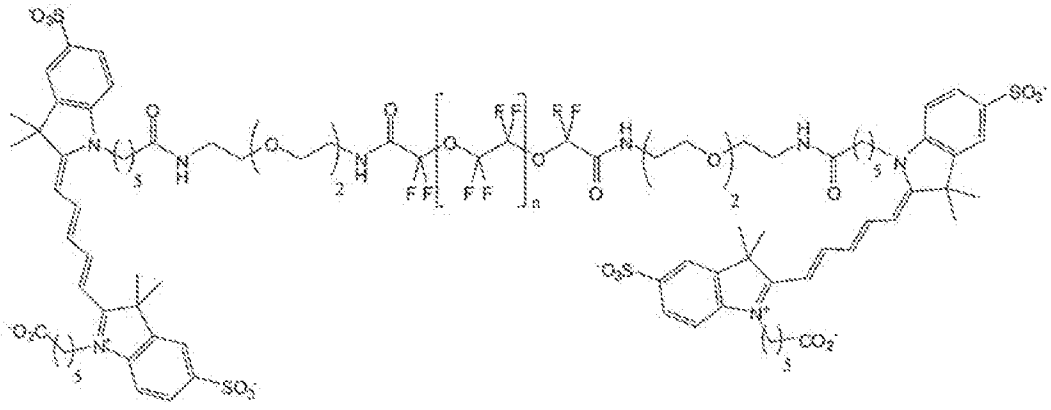


(21),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the compound of formula 28 is represented by a compound of formula 22:

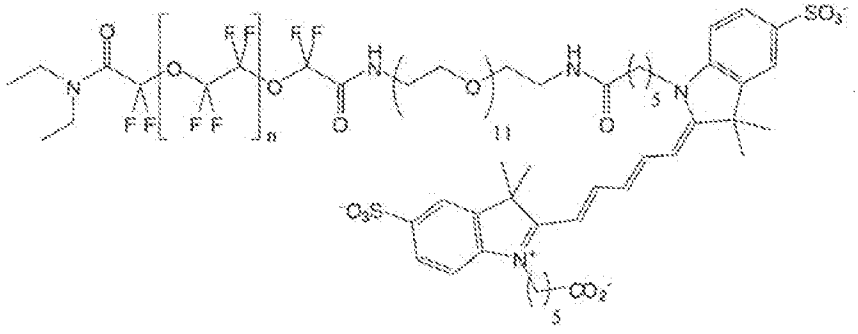


(22),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the compound of formula 29 is represented by a compound of formula 23:

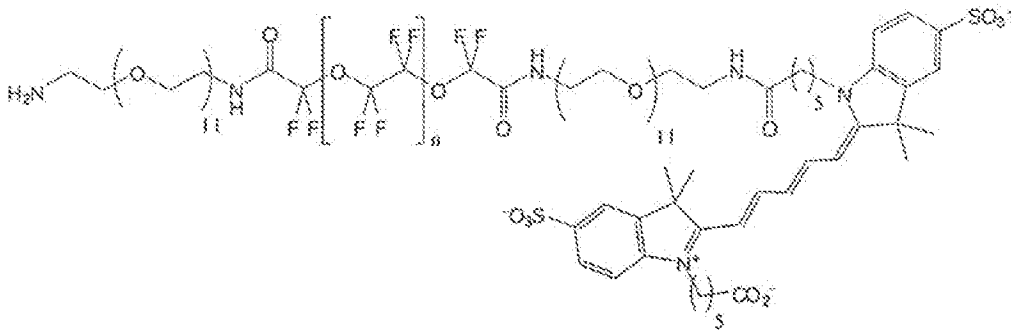


(23),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the compound of formula 30 is represented by a compound of formula 24:

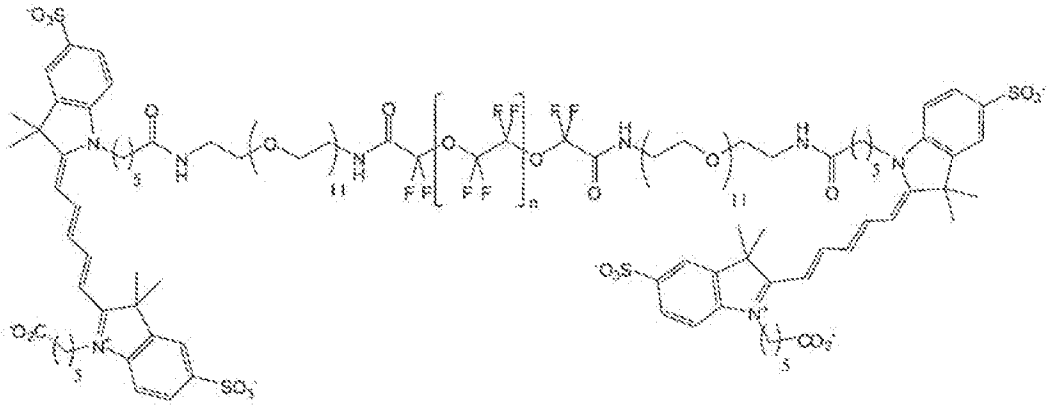


(24),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the compound of formula 31 is represented by a compound of formula 25:



(25),

wherein

n, independently for each occurrence, represents an integer from 4 to 16.

In certain embodiments, the invention provides a composition comprising two or more compounds of any one of formulae 1-37 or 40-41, e.g., with differing values of n.

The present invention provides certain compounds with a substantially different chemical shift from PFPE, including purified preparations of those compounds that may be used as imaging reagents in methods of the invention. For instance, the invention provides a compound of formula 42:

(42).

The present invention also provides novel compositions comprising combinations of the compounds recited above. For example, the present invention provides a composition comprising a compound of formula 10 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 10 and a compound of formula 1 contains the compound of formula 10 and the compound of formula 1 in a molar ratio from 1:1 to 1:100. In certain embodiments, the composition comprising a compound of formula 10 and a compound of formula 1 contain the compound of formula 10 and the compound of formula 1 in a molar ratio of 1:2.

The present invention provides a further composition comprising a compound of formula 12 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 12 and a compound of formula 1 contains the

compound of formula 12 and the compound of formula 1 in a molar ratio from 1:1 to 1:100. In certain embodiments, the composition comprising a compound of formula 12 and a compound of formula 1 contain the compound of formula 12 and the compound of formula 1 in a molar ratio of 1:4.

The present invention provides a further composition comprising a compound of formula 14 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 14 and a compound of formula 1 contains the compound of formula 14 and the compound of formula 1 in a molar ratio from 1:1 to 1:100. In certain embodiments, the composition comprising a compound of formula 14 and a compound of formula 1 contain the compound of formula 14 and the compound of formula 1 in a molar ratio of 1:10.

The present invention provides a further composition comprising a compound of formula 16 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 16 and a compound of formula 1 contains the compound of formula 16 and the compound of formula 1 in a molar ratio from 1:1 to 1:100. In certain embodiments, the composition comprising a compound of formula 16 and a compound of formula 1 contain the compound of formula 16 and the compound of formula 1 in a molar ratio of 1:100.

The present invention provides a further composition comprising a compound of formula 18 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 18 and a compound of formula 1 contains the compound of formula 18 and the compound of formula 1 in a molar ratio from 1:1 to 1:200. In certain embodiments, the composition comprising a compound of formula 18 and a compound of formula 1 contain the compound of formula 18 and the compound of formula 1 in a molar ratio of 1:50.

The present invention provides a further composition comprising a compound of formula 40 and a compound of formula 1. In certain embodiments, the composition comprising a compound of formula 40 and a compound of formula 1 contains the compound of formula 40 and the compound of formula 1 in a molar ratio from 1:1 to 1:200. In certain embodiments, the composition comprising a compound of formula 40 and a compound of formula 1 contain the compound of formula 40 and the compound of formula 1 in a molar ratio of 1:40.

The present invention provides a further composition comprising a compound of formula 16 and a compound of formula 17. In certain such embodiments, the composition further comprises a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 19 and a compound of formula 19. In certain such embodiments, the composition further comprises a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 40 and a compound of formula 41. In certain such embodiments, the composition further comprises a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 10 and a compound of formula 20.

The present invention provides a further composition comprising a compound of formula 10, a compound of formula 20, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 21.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 22.

The present invention provides a further composition comprising a compound of formula 12 and a compound of formula 23.

The present invention provides a further composition comprising a compound of formula 12, a compound of formula 23, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 24.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 25.

The present invention provides a further composition comprising a compound of formula 10 and a compound of formula 26.

The present invention provides a further composition comprising a compound of formula 10, a compound of formula 26, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 27.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 28.

The present invention provides a further composition comprising a compound of formula 12 and a compound of formula 29.

The present invention provides a further composition comprising a compound of formula 12, a compound of formula 29, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 30.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 31.

The present invention provides a further composition comprising a compound of formula 10 and a compound of formula 32.

The present invention provides a further composition comprising a compound of formula 10, a compound of formula 32, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 33.

The present invention provides a further composition comprising a compound of formula 11 and a compound of formula 34.

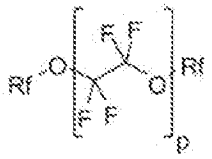
The present invention provides a further composition comprising a compound of formula 12 and a compound of formula 35.

The present invention provides a further composition comprising a compound of formula 12, a compound of formula 35, and a compound of formula 1.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 36.

The present invention provides a further composition comprising a compound of formula 13 and a compound of formula 37.

The present invention also provides compositions comprising combinations of the compounds recited above and PFPE oxide, formula 1a (Perfluoropoly(ethylene glycol) Dialkyl Ether, Exfluor Inc., TX),



PFPE oxide, 1a

wherein

p represents an integer from 8 to 13; and

Rf is $CF_3$ and $CF_2CF_3$ in a ratio of 2:1, based on 19F NMR analysis.

For example, the present invention provides a composition comprising a compound of any one of formulae 1-9 and a compound of formula 1a. In certain embodiments, the present invention provides a composition comprising a compound of formula 1 and a compound of formula 1a. As a further example, the present invention provides a composition comprising a compound of formula 10 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 14 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 14, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 16 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 16, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 18 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 18, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 40 and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 40, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 1, a compound of formula 16, a compound of formula 17, and a compound of formula 1a. The present invention provides

a further composition comprising a compound of formula 1, a compound of formula 18, a compound of formula 19, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 1, a compound of formula 40, a compound of formula 41, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 20, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 20, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 21, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 22, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 23, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 23, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 24, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 25, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 26, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 26, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 27, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 28, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 29, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 29, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 30, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a

compound of formula 31, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 32, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 32, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 33, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 34, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 35, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 35, a compound of formula 1, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 36, and a compound of formula 1a. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 37, and a compound of formula 1a. In certain embodiments of any of the foregoing, the composition comprises 80-95% v/v of a compound of formula 1a, such as 80% , 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% v/v of a compound of formula 1a.

The present invention also provides compositions comprising combinations of the compounds recited above and perfluoro-15-crown-5 ether. For example, the present invention provides a composition comprising a compound of any one of formulae 1-9 and perfluoro-15-crown-5 ether. In certain embodiments, the present invention provides a composition comprising a compound of formula 1 and perfluoro-15-crown-5 ether. The present invention further provides a composition comprising a compound of formula 1a and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 10 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 10, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 12, a compound of formula 1, and

perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 14 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 14, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 16 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 16, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 18 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 18, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 40 and perfluoro-15-crown-5 ether. As a further example, the present invention provides a composition comprising a compound of formula 40, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention further provides a composition comprising a compound of formula 16, a compound of formula 17, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention further provides a composition comprising a compound of formula 18, a compound of formula 19, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention further provides a composition comprising a compound of formula 40, a compound of formula 41, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 20, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 20, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 21, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 22, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 23, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 23, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula

13, a compound of formula 24, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 25, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 26, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 26, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 27, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 28, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 29, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 29, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 30, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 31, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 32, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 10, a compound of formula 32, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 33, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 11, a compound of formula 34, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 35, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 12, a compound of formula 35, a compound of formula 1, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 36, and perfluoro-15-crown-5 ether. The present invention provides a further composition comprising a compound of formula 13, a compound of formula 37,

and perfluoro-15-crown-5 ether. In certain embodiments of any of the foregoing, the composition comprises 80-95% v/v of perfluoro-15-crown-5 ether, such as 80% , 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% v/v of perfluoro-15-crown-5 ether.

The present invention also provides formulations of the compounds or compositions of the present invention as described above that are suitable for uptake by cells. For example, the compounds or compositions of the present invention may be formulated as an emulsion. For example, the present invention provides an emulsion comprising a compound of any one of formulae 1-37 or 40-41. The present invention further provides an emulsion comprising a composition comprising a compound of formula 10 and a compound of formula 1; a composition comprising a compound of formula 12 and a compound of formula 1; a composition comprising a compound of formula 14 and a compound of formula 1; a composition comprising a compound of formula 16 and a compound of formula 1; a composition comprising a compound of formula 18 and a compound of formula 1; a composition comprising a compound of formula 40 and a compound of formula 1; a composition comprising a compound of formula 16 and a compound of formula 17; a composition comprising a compound of formula 16, a compound of formula 17, and a compound of formula 1; a composition comprising a compound of formula 18 and a compound of formula 19; a composition comprising a compound of formula 18, a compound of formula 19, and a compound of formula 1; a composition comprising a compound of formula 40 and a compound of formula 41; a composition comprising a compound of formula 40, a compound of formula 41, and a compound of formula 1; a composition comprising a compound of formula 10 and a compound of formula 20; a composition comprising a compound of formula 10, a compound of formula 20, and a compound of formula 1; a composition comprising a compound of formula 11 and a compound of formula 21; a composition comprising a compound of formula 11 and a compound of formula 22; a composition comprising a compound of formula 12 and a compound of formula 23; a composition comprising a compound of formula 12, a compound of formula 23, and a compound of formula 1; a composition comprising a compound of formula 13 and a compound of formula 24; a composition comprising a compound of formula 13 and a compound of formula 25; a composition comprising a compound of formula 10 and a compound of formula 26; a

composition comprising a compound of formula 10, a compound of formula 26, and a compound of formula 1; a composition comprising a compound of formula 11 and a compound of formula 27; a composition comprising a compound of formula 11 and a compound of formula 28; a composition comprising a compound of formula 12 and a compound of formula 29; a composition comprising a compound of formula 12, a compound of formula 29, and a compound of formula 1; a composition comprising a compound of formula 13 and a compound of formula 30; a composition comprising a compound of formula 13 and a compound of formula 31; a composition comprising a compound of formula 10 and a compound of formula 32; a composition comprising a compound of formula 10, a compound of formula 32, and a compound of formula 1; a composition comprising a compound of formula 11 and a compound of formula 33; a composition comprising a compound of formula 11 and a compound of formula 34; a composition comprising a compound of formula 12 and a compound of formula 35; a composition comprising a compound of formula 12, a compound of formula 35, and a compound of formula 1; a composition comprising a compound of formula 13 and a compound of formula 36; or a composition comprising a compound of formula 13 and a compound of formula 37.

The present invention further provides an emulsion comprising a composition comprising a compound of any one of formulae 1-9 and a compound of formula 1a, such as a compound of formula 1 and a compound of formula 1a; a composition comprising a compound of formula 10 and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 12 and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 14 and a compound of formula 1a; a composition comprising a compound of formula 14, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 16 and a compound of formula 1a; a composition comprising a compound of formula 16, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 18 and a compound of formula 1a; a composition comprising a compound of formula 18, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 40 and a

compound of formula 1a; a composition comprising a compound of formula 40, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 16, a compound of formula 17, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 18, a compound of formula 19, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 40, a compound of formula 41, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 20, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 20, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 21, and a compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 22, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 23, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 23, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 13, a compound of formula 24, and a compound of formula 1a; a composition comprising a compound of formula 13, a compound of formula 25, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 26, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 26, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 27, and a compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 28, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 29, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 29, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 13, a compound of formula 30, and a compound of formula 1a; a composition comprising a compound of formula 13, a compound of formula 31, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 32, and a compound of formula 1a; a composition comprising a compound of formula 10, a compound of formula 32, a compound of formula 1, and a

compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 33, and a compound of formula 1a; a composition comprising a compound of formula 11, a compound of formula 34, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 35, and a compound of formula 1a; a composition comprising a compound of formula 12, a compound of formula 35, a compound of formula 1, and a compound of formula 1a; a composition comprising a compound of formula 13, a compound of formula 36, and a compound of formula 1a; or a composition comprising a compound of formula 13, a compound of formula 37, and a compound of formula 1a. In certain embodiments of any of the foregoing, the composition comprises 80-95% v/v of a compound of formula 1a, such as 80% , 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% v/v of a compound of formula 1a.

The present invention further provides an emulsion comprising a composition comprising a compound of any one of formulae 1-9 and perfluoro-15-crown-5 ether, such as a compound of formula 1 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 1a and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 14 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 14, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 16 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 16, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 18 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 18, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 40 and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 40, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 16, a compound of formula 17, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 18, a

compound of formula 19, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 40, a compound of formula 41, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 20, and perfluoro-15-crown-5 ethe; a composition comprising a compound of formula 10, a compound of formula 20, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 21, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 22, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 23, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 23, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 13, a compound of formula 24, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 13, a compound of formula 25, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 26, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 26, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 27, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 28, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 29, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 29, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 13, a compound of formula 30, and perfluoro-15-crown-5 ethe; a composition comprising a compound of formula 13, a compound of formula 31, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 32, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 10, a compound of formula 32, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 33, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 11, a compound of formula 34, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 35, and

perfluoro-15-crown-5 ether; a composition comprising a compound of formula 12, a compound of formula 35, a compound of formula 1, and perfluoro-15-crown-5 ether; a composition comprising a compound of formula 13, a compound of formula 36, and perfluoro-15-crown-5 ethe; or a composition comprising a compound of formula 13, a compound of formula 37, and perfluoro-15-crown-5 ether. In certain embodiments of any of the foregoing, the composition comprises 80-95% v/v of perfluoro-15-crown-5 ether, such as 80% , 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% v/v of perfluoro-15-crown-5 ether.

In certain embodiments, the emulsion may further comprise a block copolymer of polyethylene and polypropylene glycol. In certain embodiments, the emulsion may further comprise a Pluronic™. Nonionic Pluronic™ surfactants, polyethyleneoxide (PEO)/polypropyleneoxide (PPO)/polyethyleneoxide (PEO) block (ABA type), (PEO/PPO/PEO) block copolymers, exhibit a wide range of hydrophilicity/ hydrophobicity as a function of the PEO/PPO ratio, so that one can expect to obtain different phase separated morphologies with polymers such as PLA as well as different degrees of hydration of the matrix. In particular, hydration plays an important role in determining polymer degradation via hydrolysis of the ester backbone. These polymeric surfactants exhibited minimal toxicities in vivo and some of them are in clinical use, as described by BASF Corporation in their 1989 Technical Bulletin; Attwood, et al., Int. J. Pharm. 26,25 (1985); and U.S. Pat. No. 4,188,373 to Krezanoski. These materials can be obtained from BASF Corporation. In certain embodiments, emulsions of the present invention further comprise tri-block copolymer which comprises polyethyleneoxide and polypropyleneoxide.

In certain embodiments, emulsions of the present invention comprise a tri-block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) with an average molecular weight of 1900. In certain such embodiments, the tri-block copolymer comprises 50% PEO content with an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38. In certain such embodiments, the hydrophilic-lipophilic balance (HLB) value of the tri-block copolymer is 19, wherein the HLB value can be calculated from the following equation:

$$HLB = -36 \frac{m}{2n + m} + 33$$

where n represents the number of repeat units in the PEO segment of the polymer and m represents the number of repreat units in the PPO segment of the polymer. Exemplary tri-block copolymers can be obtained from BASF Corporation and are sold under the trade name of Pluronic™ L35.

In certain embodiments, emulsions of the present invention comprise a tri-block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) with an average molecular weight of 2900. In certain such embodiments, the tri-block copolymer comprises 40% PEO content with an average number of PEO units of about 26, such as 26.36, and an average number of PPO units of about 30, such as 30.00. In certain such embodiments, the hydrophilic-lipophilic balance (HLB) value of the tri-block copolymer is 15. Exemplary tri-block copolymers can be obtained from BASF Corporation and are sold under the trade name of Pluronic™ L64.

In certain embodiments, emulsions of the present invention comprise a tri-block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) with an average molecular weight of 8400. In certain such embodiments, the tri-block copolymer comprises 80% PEO content with an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97. In certain such embodiments, the hydrophilic-lipophilic balance (HLB) value of the tri-block copolymer is 29. Exemplary tri-block copolymers can be obtained from BASF Corporation and are sold under the trade name of Pluronic™ F68.

In certain embodiments, emulsions of the present invention comprise a tri-block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) with an average molecular weight of 6500. In certain such embodiments, the tri-block copolymer comprises 50% PEO content with an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03. In certain such embodiments, the hydrophilic-lipophilic balance (HLB) value of the tri-block copolymer is 15. Exemplary tri-block copolymers can be obtained from BASF Corporation and are sold under the trade name of Pluronic™ P105.

In certain embodiments, the emulsion may further comprise a lipid. In certain embodiments of emulsions of the present invention that further comprise a lipid, the lipid

is DMPC. In certain embodiments of emulsions of the present invention that further comprise a lipid, the emulsion further comprises a block copolymer. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38. In certain embodiments, the emulsion of the present invention further comprises DMPC and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38 (e.g., Pluronic™ L35).

In certain embodiments, the emulsion may further comprise polyethylamine.

In certain embodiments, the emulsion may further comprise protamine sulfate. In certain embodiments of emulsions of the present invention that further comprise protamine sulfate, the emulsion further comprises a block copolymer. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide). In certain such embodiments the block copolymer is poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38 (e.g., Pluronic™ L35). In certain embodiments, the emulsion of the present invention further comprises protamine sulfate and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 1900, an average number of PEO units of about 22, such as 21.59, and an average number of PPO units of about 16, such as 16.38 (e.g., Pluronic™ L35).

In certain embodiments, the emulsion may further comprise an emulsifier. In certain such embodiments, the emulsifier is also a non-ionic solubiliser. In certain embodiments, the emulsifier comprises glycerol polyethylene glycol ricinoleate. In certain such embodiments, the emulsifier further comprises fatty acid esters of polyethylene glycol, free polyethylene glycols, and ethoxylated glycerol.

In certain embodiments, the emulsifier is prepared by reacting castor oil and ethylene oxide in a molar ratio of 1:35. Exemplary emulsifiers can be obtained from BASF Corporation and are sold under the trade name of Cremophor® EL.

In certain embodiments, the invention contemplates any combination of the foregoing. Those skilled in the art will recognize that all specific combinations of the individual possible components of the emulsions as disclosed herein, e.g., a compound of any one of formulae 1-37, 40-41, 1a, or perfluoro-15-crown-5 ether, a block copolymer (e.g., a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide), a lipid, an emulsifier comprising glycerol polyethylene glycol ricinoleate, polyethylamine, or protamine sulfate, are within the scope of the invention. As an example, an emulsion may contain one or more compound(s) of any one of formulae 1-37, 40-41, 1a, or perfluoro-15-crown-5 ether, a block copolymer (e.g., a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide), a lipid, and polyethylamine and/or protamine sulfate.

In certain embodiments, the emulsion comprises a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 16, a compound of formula 17, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400,

an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 18, a compound of formula 19, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene

oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 40, a compound of formula 41, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 40, a

compound of formula 41, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97(e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 1a, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1a and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of

PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)

(PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; a compound of formula 1a, perfluoro-15-crown-5 ether, and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as

152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68); perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and protamine sulfate; perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97 (e.g., Pluronic™ F68), and polyethylamine; perfluoro-15-crown-5 ether and a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 8400, an average number of PEO units of about 153, such as 152.73, and an average number of PPO units of about 29, such as 28.97(e.g., Pluronic™ F68); a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1a, a compound of formula 1, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1a, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1a, a compound of formula 1, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an

average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1, a compound of formula 16, a compound of formula 17, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1, a compound of formula 18, a compound of formula 19, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1, a compound of formula 40, a compound of formula 41, perfluoro-15-crown-5 ether, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1, a compound of formula 16, a compound of formula 17, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of

PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); a compound of formula 1, a compound of formula 18, a compound of formula 19, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL); or a compound of formula 1, a compound of formula 40, a compound of formula 41, a compound of formula 1a, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL).

In certain embodiments, the emulsion comprises a compound of formula 42, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) tri-block copolymer with an average molecular weight of 6500, an average number of PEO units of about 74, such as 73.86, and an average number of PPO units of about 56, such as 56.03 (e.g., Pluronic™ P105), and an emulsifier, wherein the emulsifier is a non-ionic solubiliser comprising glycerol polyethylene glycol ricinoleate (e.g., Cremophor® EL).

Emulsions of the present invention will preferably have a distribution of particle sizes that allow adequate cellular uptake. In certain embodiments, a uniform particle size may be advantageous. The desired degree of uniformity of particle size may vary depending upon the application. In certain embodiments, the emulsion has a mean particle size less than 500 nm, or less than 400 nm, or less than 300 nm, or less than 200 nm in diameter. Optionally, 25%, or 50%, or 75% or more of the particles will fall within the selected range. Particle sizes may be evaluated by, for example, light scattering techniques or by visualizing the emulsion particles using EM micrographs. In certain cell types that have a relatively small amount of cytoplasm, such as most stem cells, the emulsions have a mean particle size of less than 200 nm, or less than 100 nm, or less than 50 nm in diameter.

Emulsions for use in cells should preferably be stable at a wide range of temperatures. In certain embodiments, emulsions will be stable at body temperature (37 °C for humans) and at a storage temperature, such as 4 °C or room temperature (20-25 °C). For example, it will often be desirable to store the emulsion at a cool temperature, in the range of 2 – 10 °C, such as 4 °C, and then warm the emulsion to room temperature (e.g., 18 to 28 °C, and more typically 20 to 25 °C). After labeling of cells, the emulsion will experience a temperature of about 37 °C. Accordingly, a preferred emulsion will retain the desired range of particle sizes at temperatures ranging from refrigeration temperatures up to body temperature. In certain embodiments, the emulsion is stable at temperatures ranging from 4 °C to 37 °C.

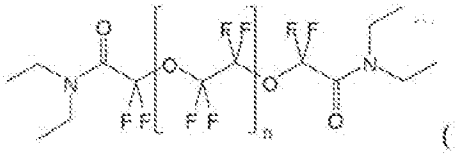
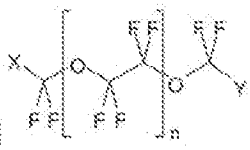
In certain embodiments, the emulsion has a polydispersity index ranging from 0.1 to 0.2.

The present invention provides a method for preparing an emulsion of a PFPE derivative with a block copolymer using low energy methods. In certain embodiments, the block copolymer is a tri-block copolymer which comprises poly(ethylene oxide) and poly(propylene oxide).

The present invention further provides a method for preparing an emulsion comprising low energy methods. In certain such embodiments, the low energy method comprises a thin film method.

In certain embodiments of emulsions of the present invention, the emulsion further comprises PFPE-oxide, 1a.

The present invention also provides a method for preparing a composition comprising a compound of formula 1 and a compound of formula 38:

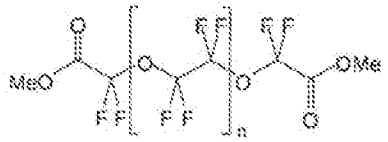

(1) and

(38),

wherein

n, independently for each occurrence, represents an integer from 4 to 16; and

one or both of X and Y is an amide other than diethyl amide,

comprising:



1) reacting perfluoropolyether methyl ester (39), (39), having two methyl ester end groups with a primary or secondary aliphatic amine other than diethyl amine;

2) reacting unmodified methyl ester end groups with excess diethyl amine; and

3) removing unreacted diethyl amine; and

4) optionally removing non-volatile amines by solid phase extraction and filtration using fluorous phase silica gel (FluoroFlash, Fluorous Inc.)

In certain embodiments, more than 80%, or more than 85%, or more than 90%, or more than 91%, or more than 92%, or more than 93%, or more than 94%, or more than 95% of the methyl ester end groups are converted to amides.

In certain embodiments, excess diethyl amine is removed *in vacuo*.

In certain embodiments, excess non volatile amine is eluted with fluorophobic solvent after loading the sample onto fluorous silica gel column FluoroFlash (Fluorous Inc.).

In certain embodiments, less than one equivalent of the amine other than diethyl amine is reacted with perfluoropolyether methyl ester 39 (e.g., less than 100 mol% of amine other than diethyl amine as compared to perfluoropolyether methyl ester 39 is reacted). In certain embodiments, less than 90 mol%, or less than 80 mol%, or less than 70 mol %, or less than 60 mol%, or less than 50 mol%, or less than 40 mol%, or less than 30 mol %, or less than 20 mol%, or less than 10 mol % of amine other than diethyl amine is reacted with perfluoropolyether methyl ester 39. In certain such embodiments, the reaction between the amine other than diethyl amine and perfluoropolyether methyl ester 39 is allowed to proceed to completion. In certain embodiments wherein any amine other than diethyl amine remains unreacted, the unreacted amine other than diethyl amine is removed by selective extraction with an organic solvent such as ethanol or THF. In certain embodiments the unreacted amine other then diethylamine is eluted from a fluorous phase column by fluorophobic solvent .

In certain embodiments, compositions prepared using the method described above are as defined above for the composition comprising a compound of formula 10 and a compound of formula 1; the composition comprising a compound of formula 12 and a

compound of formula 1; the composition comprising a compound of formula 14 and a compound of formula 1; the composition comprising a compound of formula 16 and a compound of formula 1; or the composition comprising a compound of formula 18 and a compound of formula 1; or the composition comprising a compound of formula 40 and a compound of formula 1.

Detection moieties suitable for PET imaging may also be used to create dual mode imaging reagents that are detectable by nuclear magnetic resonance techniques and by PET techniques. For example, the $^{18}F$ isotope is a potent label for PET detection methods. A fluorocarbon imaging reagent may comprise a mixture of $^{18}F$ and $^{19}F$ isotopes, thus providing a dual mode label that is suitable for MRI/MRS and PET. $^{18}F$ and $^{19}F$ may also be added in separate monomers to form a mixed copolymer, or $^{18}F$ portions may be located at either end of a linear polyether, at the position where most other functional moieties would be added. $^{18}F$ has no NMR signal and so may be added at positions that would, for example, tend to decrease NMR linewidth, simplify the NMR spectrum, or alleviate chemical shifts from resonances that adversely affect the read-out obtained by a nuclear magnetic resonance technique. In addition, molecules of the fluorocarbon imaging reagents can incorporate other radioisotopes that are effective PET probes, such as $^{11}C$, $^{15}O$, and $^{13}N$. Those skilled in the art can, in view of this specification, devise many other PET-detectable moieties that can be incorporated into or, for example, attached to an endgroup(s), of the imaging reagents of this disclosure.

The properties of an emulsion may be controlled primarily by the properties of the imaging reagent itself, the nature of surfactants and/or solvents used, and the type of processing device (e.g., sonicator, Microfluidizer, homogenizer, etc.). Methods for forming emulsions with certain PFPE molecules are extensively described in U.S. Pat. Nos. 5,330,681 and 4,990,283, herein incorporated by reference in their entirety. A continuous phase of a polyhydroxylated compound, such as polyalcohols and saccharides in concentrated aqueous solution may be effective. The following polyalcohols and saccharides have proved to be particularly effective: glycerol, xylitol, mannitol, sorbitol, glucose, fructose, saccharose, maltitol, dimer compounds of glycerol (di-glycerol or bis(2,3-di-hydroxypropyl) ether, solid water soluble polyhydroxylated compounds as sugars and glycerol condensation products as triglycerol and tetraglycerol. The dispersion in emulsion may be performed in the presence of conventional surfactants, including

cationic, anionic, amphoteric and non-ionic surfactants. Examples of suitable surfactants include sodium lauryl sulphate, sulphosuccinate (sulphosuccinic hemiester), cocoamphocarboxyglycinate, potassium cetyl phosphate, sodium alkyl-polyoxyethylene-ether carboxylate, potassium benzalconium chloride, alkyl amidopropyl betaine, cetyl-stearilic ethoxylated alcohol, and sorbitan-ethoxylate(20)-mono-oleate Tween 20. While thermodynamic equations may be used to attempt to predict mixtures of imaging reagents that will give emulsions having the desired particle sizes and stability, it is generally accepted that actual testing of various mixtures will be most effective. The emulsification of mixtures is simple and quick, permitting rapid testing of a wide range of combinations to identify those that give rise to emulsions that are suitable for use in the methods disclosed herein.

Preferably an emulsion is designed to facilitate uptake of the imaging reagent by the subject cells. A surfactant may be designed to form stable emulsions that carry a large quantity of PFPE (e.g., PFPE amide, as in compounds or compositions of the present invention) into the aqueous phase. Additionally, it may have properties that increase the intracellular delivery of the emulsion particles in the shortest possible incubation time. Increasing the PFPE (e.g., PFPE amide, as in compounds or compositions of the present invention) intracellular loading improves sensitivity to the labeled cells. Furthermore, minimizing the culture time can be important when working with the primary cells cultures. The efficiency of intracellular uptake depends on cell type. For example macrophages and some dendritic cells will endocytose almost any particulate, whereas other cell types of interest may only be weakly phagocytic. In either case the uptake efficiency can be boosted substantially by designing the surfactant so that the surface of the emulsion particle has properties that promote cellular uptake in culture (i.e. "self-delivering" emulsion particles). The emulsion particle surface can be made to have lipophilic, or optionally cationic, properties via appropriate surfactant design. For example the surfactant can incorporate lipids, such as cationic or neutral lipids, oil-in-water emulsions, micelles, mixed micelles, or liposomes, that tend to bind to or fuse with the cell's surface, thereby enhancing emulsion particle uptake. In certain embodiments, a colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (i.e., an artificial membrane vesicle). The preparation and use of such systems is well known in the art. Suitable cationic lipids are described in the following and are herein incorporated in

their entirety: Felgner et al., 1987, PNAS 84, 7413-7417; Eppstein et al., U.S. Pat. No. 4,897,355), (Rose, U.S. Pat. No. 5,279,833; Eppand et al. U.S. Pat. No. 5,283,185; Gebeyehu et al., U.S. Pat. No. 5,334,761; Nantz et al., U.S. Pat. No. 5,527,928; Bailey et al., U.S. Pat. No. 5,552,155; Jesse, U.S. Pat. No. 5,578,475). Other approaches include incorporation into the surfactant peptides (e.g. oligo-Arg9 and TAT-like peptides) that facilitate entry into cells, or antibodies that target specific cell surface molecules. Additionally, in certain embodiments, one can incorporate small cationic proteins into the surfactant, such as protamine sulfate, to enhance cellular uptake. Protamine sulfate is non-toxic to cells and has FDA approval for use in humans as a heparin antagonist. In certain embodiments, colloidal dispersion systems are used, such as macromolecule complexes, nanocapsules, microspheres, and beads. Other approaches for enhancing uptake of the emulsified fluorocarbons, such as by using additional transfection agents or by using electroporation of the cells, is described herein.

In preferred embodiments, emulsions have "self-delivering" properties without having to add uptake enhancing reagents. Said emulsions are preferably stable and have a shelf-life of a period of months or years.

It is understood that surfactants and uptake enhancing reagents are not meant to be exclusive groups and in some cases they may be overlapping.

The term "acyl" is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)-, preferably alkylC(O)-.

The term "acylamino" is art-recognized and refers to an amino group substituted with an acyl group and may be represented, for example, by the formula hydrocarbylC(O)NH-.

The term "acyloxy" is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)O-, preferably alkylC(O)O-.

The term "alkoxy" refers to an alkyl group, preferably a lower alkyl group, having an oxygen attached thereto. Representative alkoxy groups include methoxy, ethoxy, propoxy, tert-butoxy and the like.

The term "alkoxyalkyl" refers to an alkyl group substituted with an alkoxy group and may be represented by the general formula alkyl-O-alkyl.

The term "alkenyl", as used herein, refers to an aliphatic group containing at least one double bond and is intended to include both "unsubstituted alkenyls" and "substituted

alkenyls", the latter of which refers to alkenyl moieties having substituents replacing a hydrogen on one or more carbons of the alkenyl group. Such substituents may occur on one or more carbons that are included or not included in one or more double bonds. Moreover, such substituents include all those contemplated for alkyl groups, as discussed below, except where stability is prohibitive. For example, substitution of alkenyl groups by one or more alkyl, carbocyclyl, aryl, heterocyclyl, or heteroaryl groups is contemplated.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (*e.g.*, $C_1$-$C_{30}$ for straight chains, $C_3$-$C_{30}$ for branched chains), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties subetituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-CF_3$, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, $-CF_3$, -CN, and the like.

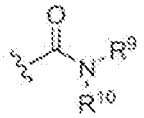
The term "$C_{x-y}$" when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups that contain from x to y carbons in the chain. For example, the term "$C_{x-y}$alkyl" refers to substituted or unsubstituted saturated hydrocarbon groups, including straight-chain alkyl and branched-chain alkyl groups that contain from x to y carbons in the chain, including haloalkyl groups such as trifluoromethyl and 2,2,2-tirfluoroethyl, etc. $C_0$ alkyl indicates a hydrogen where the group is in a terminal position, a bond if internal. The terms "$C_{2-y}$alkenyl" and "$C_{2-y}$alkynyl" refer to substituted or unsubstituted unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The term "alkylamino", as used herein, refers to an amino group substituted with at least one alkyl group.

The term "alkylthio", as used herein, refers to a thiol group substituted with an alkyl group and may be represented by the general formula alkylS-.

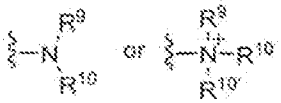
The term "alkynyl", as used herein, refers to an aliphatic group containing at least one triple bond and is intended to include both "unsubstituted alkynyls" and "substituted alkynyls", the latter of which refers to alkynyl moieties having substituents replacing a hydrogen on one or more carbons of the alkynyl group. Such substituents may occur on one or more carbons that are included or not included in one or more triple bonds. Moreover, such substituents include all those contemplated for alkyl groups, as discussed above, except where stability is prohibitive. For example, substitution of alkynyl groups by one or more alkyl, carbocyclyl, aryl, heterocyclyl, or heteroaryl groups is contemplated.

The term "amide", as used herein, refers to a group



wherein $R^9$ and $R^{10}$ each independently represent a hydrogen or hydrocarbyl group, or $R^9$ and $R^{10}$ taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines and salts thereof, *e.g.*, a moiety that can be represented by



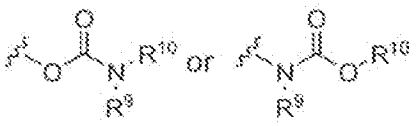
wherein $R^9$, $R^{10}$, and $R^{10'}$ each independently represent a hydrogen or a hydrocarbyl group, or $R^9$ and $R^{10}$ taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The term "aminoalkyl", as used herein, refers to an alkyl group substituted with an amino group.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group.

The term "aryl" as used herein include substituted or unsubstituted single-ring aromatic groups in which each atom of the ring is carbon. Preferably the ring is a 5- to 7-membered ring, more preferably a 6-membered ring. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is aromatic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Aryl groups include benzene, naphthalene, phenanthrene, phenol, aniline, and the like.

The term "carbamate" is art-recognized and refers to a group



wherein $R^9$ and $R^{10}$ independently represent hydrogen or a hydrocarbyl group, such as an alkyl group, or $R^9$ and $R^{10}$ taken together with the intervening atom(s) complete a heterocycle having from 4 to 8 atoms in the ring structure.

The terms "carbocycle", "carbocyclyl", and "carbocyclic", as used herein, refers to a non-aromatic saturated or unsaturated ring in which each atom of the ring is carbon. Preferably a carbocycle ring contains from 3 to 10 atoms, more preferably from 5 to 7 atoms.

The term "carbocyclylalkyl", as used herein, refers to an alkyl group substituted with a carbocycle group.

The term "carbonate" is art-recognized and refers to a group $-OCO_2-R^9$, wherein $R^9$ represents a hydrocarbyl group.

The term "carboxy", as used herein, refers to a group represented by the formula $-CO_2H$.

The term "ester", as used herein, refers to a group $-C(O)OR^9$ wherein $R^9$ represents a hydrocarbyl group.

The term "ether", as used herein, refers to a hydrocarbyl group linked through an oxygen to another hydrocarbyl group. Accordingly, an ether substituent of a hydrocarbyl group may be hydrocarbyl-O-. Ethers may be either symmetrical or unsymmetrical. Examples of ethers include, but are not limited to, heterocycle-O-heterocycle and aryl-O-heterocycle. Ethers include "alkoxyalkyl" groups, which may be represented by the general formula alkyl-O-alkyl.

The terms "halo" and "halogen" as used herein means halogen and includes chloro, fluoro, bromo, and iodo.

The terms "hetaralkyl" and "heteroaralkyl", as used herein, refers to an alkyl group substituted with a hetaryl group.

The terms "heteroaryl" and "hetaryl" include substituted or unsubstituted aromatic single ring structures, preferably 5- to 7-membered rings, more preferably 5- to 6-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms "heteroaryl" and "hetaryl" also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heteroaromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, and sulfur.

The terms "heterocyclyl", "heterocycle", and "heterocyclic" refer to substituted or unsubstituted non-aromatic ring structures, preferably 3- to 10-membered rings, more preferably 3- to 7-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms "heterocyclyl" and "heterocyclic" also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at

least one of the rings is heterocyclic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heterocyclyl groups include, for example, piperidine, piperazine, pyrrolidine, morpholine, lactones, lactams, and the like.

The term "heterocyclylalkyl", as used herein, refers to an alkyl group substituted with a heterocycle group.

The term "hydrocarbyl", as used herein, refers to a group that is bonded through a carbon atom that does not have a =O or =S substituent, and typically has at least one carbon-hydrogen bond and a primarily carbon backbone, but may optionally include heteroatoms. Thus, groups like methyl, ethoxyethyl, 2-pyridyl, and trifluoromethyl are considered to be hydrocarbyl for the purposes of this application, but substituents such as acetyl (which has a =O substituent on the linking carbon) and ethoxy (which is linked through oxygen, not carbon) are not. Hydrocarbyl groups include, but are not limited to aryl, heteroaryl, carbocycle, heterocycle, alkyl, alkenyl, alkynyl, and combinations thereof.

The term "hydroxyalkyl", as used herein, refers to an alkyl group substituted with a hydroxy group.

The term "lower" when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups where there are ten or fewer non-hydrogen atoms in the substituent, preferably six or fewer. A "lower alkyl", for example, refers to an alkyl group that contains ten or fewer carbon atoms, preferably six or fewer. In certain embodiments, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy substituents defined herein are respectively lower acyl, lower acyloxy, lower alkyl, lower alkenyl, lower alkynyl, or lower alkoxy, whether they appear alone or in combination with other substituents, such as in the recitations hydroxyalkyl and aralkyl (in which case, for example, the atoms within the aryl group are not counted when counting the carbon atoms in the alkyl substituent).

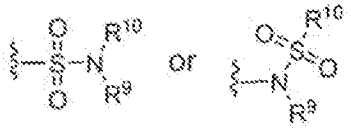
The terms "polycyclyl", "polycycle", and "polycyclic" refer to two or more rings (*e.g.*, cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls) in which two or more atoms are common to two adjoining rings, *e.g.*, the rings are "fused rings". Each of the rings of the polycycle can be substituted or unsubstituted. In certain embodiments, each ring of the polycycle contains from 3 to 10 atoms in the ring, preferably from 5 to 7.

The term "substituted" refers to moieties having substituents replacing a hydrogen on one or more carbons of the backbone. It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, *e.g.*, which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc. As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and non-aromatic substituents of organic compounds. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. Substituents can include any substituents described herein, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate.

Unless specifically stated as "unsubstituted," references to chemical moieties herein are understood to include substituted variants. For example, reference to an "aryl" group or moiety implicitly includes both substituted and unsubstituted variants.

The term "sulfate" is art-recognized and refers to the group $-OSO_3H$, or a pharmaceutically acceptable salt thereof.

The term "sulfonamide" is art-recognized and refers to the group represented by the general formulae



wherein $R^9$ and $R^{10}$ independently represents hydrogen or hydrocarbyl, such as alkyl, or $R^9$ and $R^{10}$ taken together with the intervening atom(s) complete a heterocycle having from 4 to 8 atoms in the ring structure.

The term "sulfoxide" is art-recognized and refers to the group $-S(O)-R^9$, wherein $R^9$ represents a hydrocarbyl.

The term "sulfonate" is art-recognized and refers to the group $SO_3H$, or a pharmaceutically acceptable salt thereof.

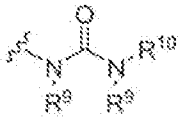
The term "sulfone" is art-recognized and refers to the group $-S(O)_2-R^9$, wherein $R^9$ represents a hydrocarbyl.

The term "thioalkyl", as used herein, refers to an alkyl group substituted with a thiol group.

The term "thioester", as used herein, refers to a group $-C(O)SR^9$ or $-SC(O)R^9$ wherein $R^9$ represents a hydrocarbyl.

The term "thioether", as used herein, is equivalent to an ether, wherein the oxygen is replaced with a sulfur.

The term "urea" is art-recognized and may be represented by the general formula



wherein $R^9$ and $R^{10}$ independently represent hydrogen or a hydrocarbyl, such as alkyl, or either occurrence of $R^9$ taken together with $R^{10}$ and the intervening atom(s) complete a heterocycle having from 4 to 8 atoms in the ring structure.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including cis- and trans-isomers, *R*- and *S*-enantiomers, diastereomers, (d)-isomers, (l)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

Methods of preparing substantially isomerically pure compounds are known in the art. If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group

cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers. Alternatively, enantiomerically enriched mixtures and pure enantiomeric compounds can be prepared by using synthetic intermediates that are enantiomerically pure in combination with reactions that either leave the stereochemistry at a chiral center unchanged or result in its complete inversion. Techniques for inverting or leaving unchanged a particular stereocenter, and those for resolving mixtures of stereoisomers are well known in the art, and it is well within the ability of one of skill in the art to choose an appropriate method for a particular situation. See, generally, Furniss *et al.* (eds.), *Vogel's Encyclopedia of Practical Organic Chemistry 5th Ed.*, Longman Scientific and Technical Ltd., Essex, 1991, pp. 809-816; and Heller, *Acc. Chem. Res.* 23: 128 (1990).

3. Cells and Labeling

Methods described herein may be used with a wide range of cells, including both prokaryotic and eukaryotic cells, and preferably mammalian cells. Technologies for cell preparation include cell culture, cloning, nuclear transfer, genetic modification and encapsulation.

A partial list of suitable mammalian cells includes: blood cells, myoblasts, bone marrow cells, peripheral blood cells, umbilical cord blood cells, cardiomyocytes (and precursors thereof), chondrocytes (cartilage cells), dendritic cells, fetal neural tissue, fibroblasts, hepatocytes (liver cells), islet cells of pancreas, keratinocytes (skin cells) and stem cells. In certain preferred embodiments, the cells to be used are a fractionated population of immune cells. Recognized subpopulations of immune cells include the lymphocytes, such as B lymphocytes (Fc receptors, MHC class II, CD19+, CD21+), helper T lymphocytes (CD3+, CD4+, CD8-), cytolytic T lymphocytes (CD3+, CD4-, CD8+), natural killer cells (CD16+), the mononuclear phagocytes, including monocytes, neutrophils and macrophages, and dendritic cells. Other cell types that may be of interest include eosinophils and basophils.

Cells may be autologous (i.e., derived from the same individual) or syngeneic (i.e., derived from a genetically identical individual, such as a syngeneic littermate or an identical twin), although allogeneic cells (i.e., cells derived from a genetically different individual of the same species) are also contemplated. Although less preferred, xenogeneic (i.e., derived from a different species than the recipient) cells, such as cells from transgenic pigs, may also be administered. When the donor cells are xenogeneic, it is preferred that the cells are obtained from an individual of a species within the same order, more preferably the same superfamily or family (e.g. when the recipient is a human, it is preferred that the cells are derived from a primate, more preferably a member of the superfamily Hominoidea).

Cells may, where medically and ethically appropriate, be obtained from any stage of development of the donor individual, including prenatal (e.g., embryonic or fetal), infant (e.g., from birth to approximately three years of age in humans), child (e.g., from about three years of age to about 13 years of age in humans), adolescent (e.g., from about 13 years of age to about 18 years of age in humans), young adult (e.g., from about 18 years of age to about 35 years of age in humans), adult (from about 35 years of age to about 55 years of age in humans) or elderly (e.g., from about 55 years and beyond of age in humans).

In many embodiments, cells are labeled by contacting the cells with an emulsion of the imaging reagent, such that the reagent is taken up by cells. Both phagocytic and non-phagocytic cells may be labeled by such a method. For example, as demonstrated in WO2005072780, both dendritic cells (phagocytic) and gliosarcoma cells (non-phagocytic) can be labeled by contacting the cells with an emulsion of the imaging reagent.

It certain aspects, a method of the invention may comprise labeling cells *in vivo* with a $^{19}F$ imaging reagent and detecting labeled cells in the subject. The cells to be labeled may be determined by specific properties of the cells such as phagocytic activity. The cells that are labeled may be controlled by the route of administration of the imaging reagent. The types of cells that are labeled may be controlled by the nature of the imaging reagent. For example, simple colloidal suspensions of imaging reagent will tend to be taken up more quickly by cells with phagocytic activity. As another example, an imaging reagent may be formulated with or covalently bound to a targeting moiety that facilitates selective targeting of the imaging reagent to a particular population of cells. In certain

embodiments, fluorocarbon imaging reagent may comprise a compound of any one of formulae 1-17, 20-37, or 40-41, or Perfluoro-15-crown ether.

In certain embodiments the cells to be labeled are stem cells. Stem cell therapies are commonly used as part of an ablative regimen for treatment of cancer with high dose radiation and/or chemotherapeutic agents. Ablative regimens generally employ hematopoietic stem cells, or populations of cells containing hematopoietic stem cells, as may be obtained, for example, from peripheral blood, umbilical cord blood or bone marrow. Cells of this type, or a portion thereof, may be labeled and tracked *in vivo* to monitor survival and engraftment at the appropriate location. Other types of stem cells are increasingly attractive as therapeutic agents for a wide variety of disorders.

As an example, cells may be mouse embryonic stem cells, or ES cells from another model animal. The labeling of such cells may be useful in tracking the fate of such cells administered to mice, optionally as part of a preclinical research program for developing embryonic stem cell therapeutics. Examples of mouse embryonic stem cells include: the JM1 ES cell line described in M. Qiu et al., Genes Dev 9, 2523 (1995), and the ROSA line described in G. Friedrich, P. Soriano, Genes Dev 5, 1513 (1991), and mouse ES cells described in US Patent No. 6,190,910. Many other mouse ES lines are available from Jackson Laboratories (Bar Harbor, Maine). Examples of human embryonic stem cells include those available through the following suppliers: Arcos Bioscience, Inc., Foster City, California, CyThera, Inc., San Diego, California, BresaGen, Inc., Athens, Georgia, ES Cell International, Melbourne, Australia, Geron Corporation, Menlo Park, California, Göteborg University, Göteborg, Sweden, Karolinska Institute, Stockholm, Sweden, Maria Biotech Co. Ltd. – Maria Infertility Hospital Medical Institute, Seoul, Korea, MizMedi Hospital – Seoul National University, Seoul, Korea, National Centre for Biological Sciences/ Tata Institute of Fundamental Research, Bangalore, India, Pochon CHA University, Seoul, Korea, Reliance Life Sciences, Mumbai, India, ReNeuron, Surrey, United Kingdom, StemCells, Inc., Palo Alto, California, Technion University, Haifa, Israel, University of California, San Francisco, California, and Wisconsin Alumni Research Foundation, Madison, Wisconsin. In addition, examples of embryonic stem cells are described in the following U.S. patents and published patent applications: 6,245,566; 6,200,806; 6,090,622; 6,331,406; 6,090,622; 5,843,780; 20020045259; 20020068045. In preferred embodiments, the human ES cells are selected from the list of approved cell lines

provided by the National Institutes of Health and accessible at http://escr.nih.gov. In certain preferred embodiments, an embryonic stem cell line is selected from the group comprising: the WA09 line obtained from Dr. J. Thomson (Univ. of Wisconsin) and the UC01 and UC06 lines, both on the current NIH registry.

In certain embodiments, a stem cell for use in disclosed methods is a stem cell of neural or neuroendocrine origin, such as a stem cell from the central nervous system (see, for example US Patent Nos. 6,468,794; 6,040,180; 5,753,506; 5,766,948), neural crest (see, for example, US Patent Nos. 5,589,376; 5,824, 489), the olfactory bulb or peripheral neural tissues (see, for example, Published US Patent Applications 20030003574; 20020123143; 20020016002 and Gritti et al. 2002 J Neurosci 22(2):437-45), the spinal cord (see, for example, US Patent Nos. 6,361,996, 5,851,832) or a neuroendocrine lineage, such as the adrenal gland, pituitary gland or certain portions of the gut (see, for example, US Patent Nos. 6,171,610 and PC12 cells as described in Kimura et al. 1994 J. Biol. Chem. 269: 18961-67). In preferred embodiments, a neural stem cell is obtained from a peripheral tissue or an easily healed tissue, thereby providing an autologous population of cells for transplant.

Hematopoietic or mesenchymal stem cells may be employed in certain disclosed methods. Recent studies suggest that bone marrow-derived hematopoietic (HSCs) and mesenchymal stem cells (MSCs), which are readily isolated, have a broader differentiation potential than previously recognized. Purified HSCs not only give rise to all cells in blood, but can also develop into cells normally derived from endoderm, like hepatocytes (Krause et al., 2001, Cell 105: 369-77; Lagasse et al., 2000 Nat Med 6: 1229-34). Similarly, HSCs from peripheral blood and from umbilical cord blood are expected to provide a useful spectrum of developmental potential. MSCs appear to be similarly multipotent, producing progeny that can, for example, express neural cell markers (Pittenger et al., 1999 Science 284: 143-7; Zhao et al., 2002 Exp Neurol 174: 11-20). Examples of hematopoietic stem cells include those described in US Patent Nos. 4,714,680; 5,061,620; 5,437,994; 5,914,108; 5,925,567; 5,763,197; 5,750,397; 5,716,827; 5,643,741; 5,061,620. Examples of mesenchymal stem cells include those described in US Patent Nos. 5,486,359; 5,827,735; 5,942,225; 5,972,703, those described in PCT publication nos. WO 00/53795; WO 00/02654; WO 98/20907, and those described in Pittenger et al. and Zhao et al., supra.

Stem cell lines are preferably derived from mammals, such as rodents (e.g. mouse or rat), primates (e.g. monkeys, chimpanzees or humans), pigs, and ruminants (e.g. cows, sheep and goats), and particularly from humans. In certain embodiments, stem cells are derived from an autologous source or an HLA-type matched source. For example, stem cells may be obtained from a subject in need of pancreatic hormone-producing cells (e.g. diabetic patients in need of insulin-producing cells) and cultured to generate autologous insulin-producing cells. Other sources of stem cells are easily obtained from a subject, such as stem cells from muscle tissue, stem cells from skin (dermis or epidermis) and stem cells from fat.

In some preferred embodiments, cells for administration to a human should be compliant with good tissue practice guidelines set by the U.S. Food and Drug Administration (FDA) or equivalent regulatory agency in another country. Methods to develop such a cell line may include donor testing, and avoidance of exposure to non-human cells and products.

Cells derived from a donor (optionally the patient is the donor) may be administered as unfractionated or fractionated cells, as dictated by the purpose of the cells to be delivered. Cells may be fractionated to enrich for certain cell types prior to administration. Methods of fractionation are well known in the art, and generally involve both positive selection (i.e., retention of cells based on a particular property) and negative selection (i. e., elimination of cells based on a particular property). As will be apparent to one of skill in the art, the particular properties (e.g., surface markers) that are used for positive and negative selection will depend on the desired population of cells. Methods used for selection/enrichment of cells may include immunoaffinity technology or density centrifugation methods. Immunoaffinity technology may take a variety of forms, as is well known in the art, but generally utilizes an antibody or antibody derivative in combination with some type of segregation technology. The segregation technology generally results in physical segregation of cells bound by the antibody and cells not bound by the antibody, although in some instances the segregation technology which kills the cells bound by the antibody may be used for negative selection.

Any suitable immunoaffinity technology may be utilized for selection/enrichment of the selected cells to be used, including fluorescence-activated cell sorting (FACS), panning, immunomagnetic separation, immunoaffinity chromatography, antibody-

mediated complement fixation, immunotoxin, density gradient segregation, and the like. After processing in the immunoaffinity process, the desired cells (the cells bound by the immunoaffinity reagent in the case of positive selection, and cells not bound by the immunoaffinity reagent in the case of negative selection) are collected and either subjected to further rounds of immunoaffinity selection/enrichment, or reserved for administration to the patient.

Immunoaffinity selection/enrichment is typically carried out by incubating a preparation of cells comprising the desired cell type with an antibody or antibody-derived affinity reagent (e.g., an antibody specific for a given surface marker), then utilizing the bound affinity reagent to select either for or against the cells to which the antibody is bound. The selection process generally involves a physical separation, such as can be accomplished by directing droplets containing single cells into different containers depending on the presence or absence of bound affinity reagent (FACS), by utilizing an antibody bound (directly or indirectly) to a solid phase substrate (panning, immunoaffinity chromatography), or by utilizing a magnetic field to collect the cells which are bound to magnetic particles via the affinity reagent (immunomagnetic separation). Alternately, undesirable cells may be eliminated from the preparation using an affinity reagent which directs a cytotoxic insult to the cells bound by the affinity reagent. The cytotoxic insult may be activated by the affinity reagent (e.g., complement fixation), or may be localized to the target cells by the affinity reagent (e.g., immunotoxin, such as ricin B chain).

Although it is expected that methods disclosed herein will be frequently used for *in vivo* monitoring of cells, it should be noted that the methodologies are equally effective for the monitoring of cells in culture, in a tissue sample or other *ex vivo* cellular material. For therapeutic uses, cells may be labeled at a desired step during the preparation for administration to the patient.

A variety of methods may be used to label cells with imaging reagent. In general, cells will be placed in contact with imaging reagent such that the imaging reagent becomes associated with the cell. Conditions will often be standard cell culture conditions designed to maintain cell viability. The term "associated" is intended to encompass any manner by which the imaging reagent and cell remain in sufficiently close physical proximity for a sufficient amount of time as to allow the imaging reagent to provide useful information about the position of the cell, whether *in vivo* or *in vitro*. Imaging reagent may be located

intracellularly, e.g. after phagocytosis or surfactant mediated entry into the cell. Immune cells, such as dendritic cells, macrophages and T cells are often highly phagocytic and data presented herein and in other studies demonstrate that such cells, and other phagocytic cell types, are readily labeled. Other cell types, such as stem cells may also be labeled, regardless of phagocytic activity. Imaging reagent may be inserted into a cell membrane or covalently or non-covalently bound to an extracellular component of the cell. For example, certain linear fluorocarbons described herein may be derivatized to attach one or more targeting moiety. A targeting moiety will be selected to facilitate association of the imaging reagent with the cell to be labeled. A targeting moiety may be designed to cause non-specific insertion of the fluorocarbon into a cell membrane (e.g., a hydrophobic amino acid sequence or other hydrophobic moiety such as a palmitoyl moiety or myristoyl moiety) or to facilitate non-specific entry into the cell. A targeting moiety may bind to a cell surface component, as in the case of receptor ligands. A targeting moiety may be a member of a specific binding pair, where the partner is a cell surface component. The targeting moiety may be, for example, a ligand for a receptor, or an antibody, such as a monoclonal or polyclonal antibody or any of the various polypeptide binding agents comprising a variable portion of an immunoglobulin (e.g., Fv fragment, single chain Fv (scFv) fragment, Fab' fragment, F(ab')2 fragment, single domain antibody, camelized antibody, humanized antibody, diabodies, tribodies, tetrabodies). In certain embodiments, fluorocarbon imaging reagent may comprise a compound of any one of formulae 1-17, 20-37, or 40-41, or Perfluoro-15-crown ether.

Cellular labeling with fluorocarbons emulsions can also be facilitated using transfection agents to aid in cell delivery. Often transfection agents consist of cationic lipids, cationic liposomes, poly-cations, and the like. The transfection agent is pre-mixed with the fluorocarbon emulsion labeling agent, whereby it becomes associated with, or coats, the emulsion particles. The transfection agent-treated emulsion particles are then added to the cultured cells and incubated so that the cells become labeled. Common transfection agents include Lipofectamine (Invitrogen, Inc) FuGene, DOTAP (Roche Diagnostics, Inc.), and poly-L-lysine. Small proteins can also be used as transfection agents, such as many types of protamines. Protamines, the major DNA-binding proteins in the nucleus of sperm in most vertebrates, package the DNA in a volume less than 5% of a somatic cell nucleus. Protamines are simple proteins of low molecular weight that are rich

in arginine and strongly basic. Commercially available protamines come from the sperm of salmon and certain other species of fish. The term "protamine" as used herein, refers to a low molecular weight cationic, arginine-rich polypeptide. The protamine molecule typically comprises about 20 to about 200 amino acids and is generally characterized by containing at least 20%, 50% or 70% arginine. Protamines are often formulated as salts, with one or more counter ions such as sulfate, phosphate and chloride.

Data provided in this application show that protamines (e.g., protamine sulfate) are highly effective in delivering PFPE fluorocarbon emulsion particles to cultured cells. Suitable protamine sulfates can come from a variety of sources (e.g., salmon, herring, trout, etc.) and be of various grades and forms (e.g., USP, grades II, III, X, etc.), with and without histones or any recombinant derivative. Examples of other protamine solutions that may be used as transfection agents include protamine phosphate, protamine chloride, protamine sulfate-2, protamine sulfate-3, protamine sulfate-10, and protamine free base.

Data provided in this application shows self-deliverable nanoemulsions prepared with fluorocarbon imaging reagents (e.g., a compound of any one of formulae 1-17, 20-37, or 40-41) and incorporate either PEI or Protamine Sulfate, optionally with a block copolymer (Pluronic™) surfactant. Simple co-incubation of cells with certain self-deliverable nanoemulsions provides sufficient cell labeling for imaging, without the need for transfection reagents.

Cell electroporation can also be used to deliver fluorocarbon emulsion particles into cells. Electroporation has the advantage that labeling is very rapid process, and it does not require the use of transfection agents. Many methods of cell electroporation are know in the art for a wide range of cell types, and several commercially available electropration instruments are available (e.g., BTX, Inc., Harvard Apparatus, Inc., Amaxa Biosystems, Inc., etc. ). Electroporation is used to deliver nucleic acids, molecules, and small particulates into cells *in vitro*. Magnetoelectorporation has been shown to be effective for MRI in cell culture (Walczak P., Magn Reson Med. 2005. Oct;54(4):769-74). Data presented in US provisional application No. 60/792003 demonstrated that cell electroporation is effective in delivering linear PFPE fluorocarbon emulsion particles into dendritic cells, and there is no barrier to using the same method to fluorocarbon-label many other phagocytic and non-phagocytic cell types, such as stem cells.

Where cells are to be used in a therapeutic regimen, various methods have been used to for delivery of cells including injections and use of special devices to implant cells in various organs. The present invention is not tied to any particular delivery method. Data presented herein demonstrate that labeled cells may be monitored regardless of whether the cells are delivered directly to a particular site or delivered systemically. For example, labeled DCs were successfully imaged following either a focal implantation directly into tissues or an intravenous injection, and T-cells were imaged following intraperitoneal injection. Cells may be inserted into a delivery device which facilitates introduction by injection or implantation into the subjects. Such delivery devices may include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the disclosure can be introduced into the subject at a desired location. The cells may be prepared for delivery in a variety of different forms. For example, the cells may be suspended in a solution or gel or embedded in a support matrix when contained in such a delivery device. Cells may be mixed with a pharmaceutically acceptable carrier or diluent in which the cells of the disclosure remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the disclosure may be prepared by incorporating cells as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

4. Nuclear Magnetic Resonance Techniques

As described herein, nuclear magnetic resonance techniques may be used to detect populations of labeled cells. The term "detect" is used to include any effort to ascertain the presence or absence of a labeled molecule or cell, particularly by a nuclear magnetic resonance technique. The term "detect" is also intended to include more sophisticated measurements, including quantitative measurements and two- or three-dimensional image generation. For example, MRI may be used to generate images of such cells. In many

instances, the labeled cells may be administered to a living subject. Following administration of the cells, some portion of the subject, or the entire subject, may be examined by MRI to generate an MRI data set. A "data set", as the term is used herein, is intended to include raw data gathered during magnetic resonance probing of the subject material, the acquisition parameters, as well as information processed, transformed or extracted from the raw data. The raw data includes transient signals obtained by MRI/MRS, including the free-induction decays, spin-echoes, stimulated-echoes, and/or gradient echoes. Examples of processed information include two-dimensional or three-dimensional pictorial representations of the subject material. The processed information may also include magnitude images, the real and imaginary image components, as well as the associated phase map images. Another example of extracted information is a score representing the amount or concentration of imaging reagent or $^{19}F$ signal in the subject material. By using the amount of $^{19}F$ signal in the subject material, and a calibration of the mean amount of imaging reagent per cell pre-implantation, one can estimate the absolute number of cells in the subject material. The amount of $^{19}F$ signal present in a subject material can be represented or calculated in many ways; for example, the average signal-to-noise-ratio (SNR) of the $^{19}F$ signal for a region of interest (ROI) may be measured and used to calculate the abundance of labeled cells. In certain embodiments, the average intensity, or pixel- or voxel-wise summation of the $^{19}F$ signal may be used to calculate the abundance of labeled cells. This type of data may be gathered at a single region of the subject, such as, for example, the spleen or another organ of particular relevance to the labeled cells. Labeled cells may be examined in contexts other than in the subject. It may be desirable to examine labeled cells in culture. In certain embodiments, labeled cells may be applied to or generated within a tissue sample or tissue culture, and labeled cells may therefore be imaged in those contexts as well. For example, an organ, tissue or other cellular material to be transplanted may be contacted with an imaging reagent to generate labeled cells prior to implantation of such transplant in a subject.

In general, labeling agents of the disclosure are designed for use in conventional MRI detection systems. In the most common implementation of MRI, one observes the hydrogen nucleus (proton, $^{1}H$) in molecules of mobile water contained in subject materials. To detect labels disclosed herein, an alternate nucleus is detected, $^{19}F$. $^{19}F$ MRI has only slightly less intrinsic sensitivity compared to $^{1}H$; the relative sensitivity is approximately

0.83. Both have a nuclear spin of +1/2. The natural isotopic abundance of $^{19}F$ is 100%, which is comparable to 99.985% for $^{1}H$. The physical principles behind the detection and image formation are the same for both $^{1}H$ and $^{19}F$ MRI. The subject material is placed in a large static magnetic field. The field tends to align the magnetic moment associated with the $^{1}H$ or $^{19}F$ nuclei along the field direction. The nuclei are perturbed from equilibrium by pulsed radio-frequency (RF) radiation at the Larmor frequency, which is a characteristic frequency proportional to the magnetic field strength where nuclei resonantly absorb energy. Upon removing the RF, the nuclei induce a transient voltage in a receiver antenna; this transient voltage constitutes the nuclear magnetic resonance (NMR) signal. Spatial information is encoded in both the frequency and/or phase of the NMR signal by selective application of magnetic field gradients that are superimposed onto the large static field. The transient voltages are generally digitized, and then these signals may be processed by, for example, using a computer to yield images.

At constant magnetic field strength, the Larmor frequency of $^{19}F$ is only slightly lower (~6 %) compared to $^{1}H$. Thus, it is straightforward to adapt conventional MRI scanners, both hardware and software, to acquire $^{19}F$ data. The $^{19}F$ detection may be coupled with different types of magnetic resonance scans, such as MRI, MRS or other techniques. Typically, it will be desirable to obtain a $^{1}H$ MRI image to compare against the $^{19}F$ image. In a living organism or other biological tissue, the proton MRI will provide an image of the subject material and allow one to define the anatomical context of the labeled cells detected in the $^{19}F$ image. In a preferred embodiment of the disclosure, data is collected for both $^{19}F$ and $^{1}H$ during the same session; the subject is not moved during these acquisitions to better ensure that the two data sets are in spatial registration. Normally, $^{19}F$ and $^{1}H$ data sets are acquired sequentially, in either order. An RF coil (i.e. antenna) can be constructed that can be electrically tuned from the $^{19}F$ and $^{1}H$ Larmor frequency. Tuning between these two frequencies can be performed manually (e.g. via an electro-mechanical variable capacitor or inductor), or electrically, via active electronic circuitry. Alternatively, with appropriate modifications to the hardware and/or software of the MRI instrument, both data sets can be acquired simultaneously, for example, to conserve imaging time. Simultaneous acquisition of the $^{19}F$ and $^{1}H$ data sets require an RF coil or antenna that can be electrically tuned simultaneously to the $^{19}F$ and $^{1}H$ Larmor frequency (i.e., a double-tuned coil). Alternatively the RF coil can be "broadband," with

one broadly-tuned electrical resonance that covers both Larmor frequencies (i.e. $^{19}F$ and $^{1}H$). Other imaging techniques, such as fluorescence detection may be coupled with $^{19}F$ MRI. This will be particularly desirable where a fluorocarbon imaging reagent has been derivatized with a fluorescent moiety. In other embodiments, the $^{19}F$ MRI scan may be combined with a PET scan in the same subject or patient by using dual-model radioactive $^{18}F/^{19}F$ fluorocarbon labeling reagents as described herein.

MRI examination may be conducted according to any suitable methodology known in the art. Many different types of MRI pulse sequences, or the set of instructions used by the MRI apparatus to orchestrate data collection, and signal processing techniques (e.g. Fourier transform and projection reconstruction) have been developed over the years for collecting and processing image data (for example, see *Magnetic Resonance Imaging, Third Edition*, editors D.D. Stark and W.G. Bradley, Mosby, Inc., St. Louis MO 1999). The reagents and methods of this disclosure are not tied to any particular imaging pulse sequence or processing method of the raw NMR signals. For example, MRI methods that can be applied to this disclosure broadly encompasses spin-echo, stimulated-echo, gradient-echo, free-induction decay based imaging, and any combination thereof. Fast imaging techniques, where more than one line in k-space or large segments of k-space are acquired from each excited signal, are also highly suitable to acquire the $^{19}F$ (or $^{1}H$) data. Examples of fast imaging techniques include fast spin-echo approaches (e.g. FSE, turbo SE, TSE, RARE, or HASTE), echo-planar imaging (EPI), combined gradient-echo and spin-echo techniques (e.g. GRASE), spiral imaging, and burst imaging. The development of new and improved pulse sequence and signal processing methods is a continuously evolving field, and persons skilled in the art can devise multiple ways to image the $^{19}F$ labeled cells in their anatomical context.

As another example of a nuclear magnetic resonance technique, MRS can be used to detect the presence of fluorocarbon-labeled cells in localized tissues or organs. Normally MRS methods are implemented on a conventional MRI scanner. Often the localized volume of interest (VOI) is defined within a conventional anatomical $^{1}H$ MRI scan. Subsequently, the magnitude of the $^{19}F$ NMR signal observed within the VOI is directly related to the number of labeled cells, and/or the mean concentration of PFPE per cell present in the tissue or organ. Methods for isolating a VOI within a much larger

subject are well known the art (for example, *Magnetic Resonance Imaging, Third Edition*, Chapter 9, Editors D.D. Stark and W.G. Bradley, Mosby, Inc., St. Louis MO 1999). Examples include using a localized RF surface coil near the VOI, surface spoiling, surface coil $B_1$-gradient methods, slice-selective $B_0$-gradient techniques, STEAM, PRESS, image selective *in vivo* spectroscopy (ISIS), and magnetic resonance spectroscopic imaging (MRSI). The development of new and improved pulse sequence and signal processing methods is continuously evolving for MRS, and persons skilled in the art can devise multiple ways to detect the $^{19}F$ NMR signals emanating from the fluorocarbon labeled cells in VOIs.

In certain embodiments the disclosure provides a method of quantifying the numbers of labeled cells *in vivo* or in subject materials within an ROI. An ROI may include all labeled cells in a subject or labeled cells in specific organs such as the pancreas, specific tissues such as lymph nodes, or any region or of one or more voxels showing detectable MRI/MRS $^{19}F$ signal. A ROI can be an otherwise undefined area beyond a particular experiment. There are a number of ways that labeled cells may be quantified in the subject materials or *in vivo*, as described herein.

Calibrating the mean "cellular dose" of $^{19}F$ labeling agent pre-implantation of a particular cell population is often a pre-requisite for quantitative cell determinations in subject materials or the patient. It is anticipated that different cell types have different innate abilities to take up the labeling agents *in vitro*, and thus the cellular dose of the labeling agent will also vary. Furthermore, different cells of the same type acquired from different sources (e.g., different patients) may have different affinities for the labeling agent. Thus a cellular dose calibration may be required. This calibration may be used, initially, to modify the labeling protocol (i.e., incubation conditions, duration of time that cells are incubated with labeling fluorocarbon emulsion, concentration of fluorocarbon emulsion in culture medium during labeling, etc.) to achieve a certain range of cellular dose before labeled cells are actually used in a subject to be imaged. Alternatively, one can fix the labeling conditions and protocol and measure the mean value $^{19}F$ labeled per cell, as is, for subsequent quantification in the subject to be imaged. In certain embodiments the mean number of $^{19}F$ molecules (F's) per cell of a labeled cell population is measured (i.e., calibrated) *in vitro* prior to administration of the cells to the subject or patient. In certain

embodiments the mean number of $^{19}F$ molecules (F's) per cell of a labeled cell population is calibrated in a test population of cells of a particular type, not necessarily destined for a patient, but used to calibrate cellular dose of labeling agent as a consequence of a particular labeling protocol or set of conditions; optionally, the value of cellular dose is then used for future labeling and *in vivo* imaging experiments in the same population type of cells with the same labeling protocol.

The cellular dose of labeling agent can be assayed *in vitro* using a variety of quantitative techniques. For example, one can use a one-dimensional (1D) $^{19}F$ NMR spectrum obtained from a cell pellet, cell suspension, or cell lysate, of a known number of labeled cells. From this spectrum, one can calculate the integrated area of the $^{19}F$ spectrum or a portion thereof, originating from the labeling reagent associated with the cells. The integrated area of the $^{19}F$ spectrum, denoted $S_{cells}$, is directly proportional to the total amount of $^{19}F$ in the cell pellet, suspension, or lysate. To measure the absolute number of $^{19}F$ nuclei, the measured $S_{cells}$ may be normalized to a $^{19}F$ standard. A $^{19}F$ standard can be, for example, a solution of a known volume and concentration of a fluoro-chemical, where one can calculate the total number of $^{19}F$ nuclei in the standard, denoted $F_{stan}$. A suitable fluoro-chemical reference ideally has a simple $^{19}F$ NMR spectrum, preferable with a single narrow resonance (e.g. trifluoroacetic acid or TFA) and optionally a $^{19}F$ chemical shift that is significantly different than the labeling fluorocarbon. The $^{19}F$ standard can be placed in the same NMR tube as the labeled cell material being measured, in a separate tube, or optionally can be measured in a separate experiment using the same NMR instrument. The integrated area of the spectrum from the $^{19}F$ standard, denoted $S_{stan}$, can then be measured. Subsequently, the mean number of $^{19}F$ per labeled cell, denoted $F_c$, can be calculated, for example using the formula:

$$F_c = \frac{S_{cells}}{S_{stan}} F_{stan} \frac{1}{N_{cells}}$$

where $N_{cells}$ is the number of labeled cells contained in the *in vitro* test sample. Quantitative NMR methods for $^{19}F$ and other nuclei are well know in the art, and those skilled can devise many variations to the cellular dose calibration procedure described above. Besides $^{19}F$ NMR, there are other quantitative methods that can be used to assay the cellular dose of the labeling reagent. For example, a reagent may be labeled fluorescently, luminescently, optically, or radioactively.

In order to extract accurate quantification of labeled cells from the $^{19}$F MRI/MRS data sets, additional calibrations and standards may be employed. For example, one can use a calibrated external $^{19}$F reference (i.e. phantom) during the actual $^{19}$F MRI/MRS scan of the subject material containing labeled cells. The image intensity of the calibrated phantom is used when analyzing the $^{19}$F MRI/MRS data set to proved an absolute standard for the number of $^{19}$F nuclei when examining the subject material or patient. The calibrated phantom is used to normalize the sensitivity of the particular MRI/MRS system that has been loaded with a particular subject to be imaged. The $^{19}$F reference may be, for example, one or more vessels containing a solution of a known concentration of $^{19}$F nuclei. In preferred embodiments, the solution contains a dilute concentration of the emulsified fluorocarbon labeling reagent. Optionally, the solution contains non-emulsified fluorocarbon labeling reagent, a gel, or liquid, for example that has been diluted in a suitable solvent. Optionally, the solution can be comprised of another fluoro-chemical, ideally with a simple $^{19}$F NMR spectrum, preferable with a single narrow NMR resonance (e.g. trifluoroacetic acid (TFA) or trifluoroacetamide (TFM) and other fluorinated acids, trifluorotoluene or trifluoroethanol). In preferred embodiments, the T1 and T2 values of the reference solution are similar to those of the labeling reagent. Optionally, the solution can contain perfluorocarbon-labeled cells, or lysates of the same. The non-cellular reference has the advantage of longer storage times. Optionally, the solution can take the form of a gel. The vessel containing the solution is preferably sealable, and can take a variety of geometries; preferred vessel geometries include ellipsoidal, cylindrical, spherical, and parallel piped shapes. One or more vessels containing $^{19}$F reference solution can be used during the $^{19}$F MRI/MRS of the subject material. If multiple $^{19}$F references (i.e. vessels) are used they can contain the same $^{19}$F concentration or different concentrations, and in the case of the latter, they ideally contain graded concentrations of fluorochemical. The placement of the calibrated $^{19}$F reference vessel(s) can be placed preferably externally or alongside, or optionally inside, the imaged subject or patient prior to data acquisition. In preferred embodiments, the reference is imaged using $^{19}$F MRI along with the subject in the same image field of view (FOV). Optionally, $^{19}$F MRS data is acquired in the reference either sequentially or in parallel with the subject data set. Optionally, data from the reference can be acquired using MRI/MRS acquired in a separate scan. Optionally, the external reference is not scanned along with a subject in every $^{19}$F

MRI/MRS examination, but rather, values of the reference $^{19}F$ signal intensity acquired using MRI/MRS is used from a scan of a comparable subject or a simulated-subject. In a given $^{19}F$ MRI/MRS scan, the calibrated $^{19}F$ standard may be sampled by one or more voxels. The observable $^{19}F$ intensity produced by a voxel may be proportional to the concentration of the fluorochemical in the solution (or gel) and the voxel volume. Often in a $^{19}F$ MRI scan the reference standard is comprised of many voxels. Often one calculates the mean intensity of one, several, or all voxels in the reference standard. Optionally, the mean image intensity is calculated over an ROI defined within the $^{19}F$ image of the reference standard. Optionally, the physical geometry of the reference standard vessel contributes to defining the observed $^{19}F$ signal intensity; for example, the volume compartment(s) containing the $^{19}F$ reference solution is smaller than the voxel volume. In other embodiments, the calibrated external reference relies on a solution with a $^{1}H$ signal intensity of a known number of detectable $^{1}H$; in this case the sensitivity of the $^{19}F$ signal in the subject material is reference to a $^{1}H$ calibrated standard. Ideally the solution or gel in the $^{1}H$ calibrated reference (contained in a vessel as described above) yields a simple $^{1}H$ NMR spectrum, preferable with a single narrow NMR resonance (e.g., $H_2O$, or mixtures of $H_2O$-$D_2O$). Other than a different nuclei, the use of the $^{1}H$ standard reference is the same in many other respects as described above for the $^{19}F$ reference. Optionally, the calibrated reference standard contains any other MRI/MRS-active nuclei. In some embodiment, the reference is an internal organ or tissue detected via $^{1}H$ MRI/MRS, where the data may be raw or normalized. In other embodiments, the reference is a standard that is not scanned with the subject, but is calibrated by relevant factors such as the weight of the patient or the size of the body cavity.

By computationally manipulating or combining two or more key parameters from the $^{19}F$ MRI/MRS data set, one can calculate the number of labeled cells present in an ROI as described herein. For example, a key set of parameters may include: (i) the cellular dose of labeling agent (i.e., $F_c$) measured *in vitro*; (ii) *in vivo* $^{19}F$ MRI/MRS data set taken in the subject at one or more time points following labeled cell administration; (iii) the voxel volume; (iv) the in-plane voxel area (i.e., area of the image pixel); (v) optionally, the MRI/MRS data set from the $^{19}F$ reference standard; (vi) optionally, the measured Johnson noise of the $^{19}F$ MRI/MRS data in the subject material; (vii) optionally, the measured signal-to-noise ratio (SNR) of one or more voxels of the $^{19}F$ MRI/MRS data set in the

subject material; (viii) optionally, the measured SNR of one or more voxels of the $^{19}F$ MRI/MRS data set from the reference standard; (ix) optionally, the $^{19}F$ NMR relaxation times (T1, T2, and T2*) of the subject material; (x) optionally, the $^{19}F$ NMR relaxation times (T1, T2, and T2*) of the reference standard (for example, see *Magnetic Resonance Imaging, Third Edition*, chapter 4, editors D.D. Stark and W.G. Bradley, Mosby, Inc., St. Louis MO 1999). Those skilled in the art can derive other parameters, combinations of the above set, or derivations thereof, particularly from the $^{19}F$ MRI/MRS dataset, that can be used to quantify the number of labeled cells *in situ*. In certain embodiments the above set of key parameters can be used to derive quantitative or statistical measures of the accuracy or confidence of the measured number of labeled cells.

There are many ways to combine the key parameters (i-x, above), any subsets of these, or any of their combinations or approximations, to estimate the effective number of labeled cells seen by $^{19}F$ MRI in the subject material, denoted by $N_c$. For example, one can use an equation of the form

$$N_c = \frac{[F_R]v}{I_R}\frac{1}{F_c}\sum_{i=1}^{N_{ROI}} I_c^{(i)}$$

where: $N_c$ = total number of labeled cells in the ROI; $[F_R]$ = concentration of $^{19}F$ in the calibrated $^{19}F$ reference solution (or gel); v = voxel volume; $I_R$ = mean intensity of the calibrated $^{19}F$ reference taken with the MRI/MRS scan, averaged over one or more voxels; $F_c$ = average $^{19}F$ cellular dose of the labeling agent measured *in vitro*; $N_{ROI}$ = number of voxels in the ROI containing labeled cells; $I_c^{(i)}$ = image intensity of the $i^{th}$ voxel in the ROI containing labeled cells; i = unitless index for voxels in the ROI containing labeled cells.

There are also many ways to approximate $N_c$ from the $^{19}F$ data set. For example, one could use the expression

$$N_c \approx \frac{I_c^{avg}}{I_R}[F_R]v\frac{1}{F_c}N_{ROI}$$

where $I_c^{avg}$ is the average intensity of the ROI containing the labeled cells, (i.e. the average intensity of the $N_{ROI}$ voxels). As another example, one could use

$$N_c \approx \frac{I_c^{avg}}{I_R} V_c \frac{1}{F_c} [F_R]$$

where $V_c$ is the total volume of the ROI containing the labeled cells. As a further example, one could use

$$N_c \approx \frac{I_c^{avg}}{I_R} \frac{V_c}{V_R} \frac{1}{F_c} N_R$$

where $V_R$ is the effective volume of the reference in the $^{19}F$ MRI/MRS and $N_R$ is the number $^{19}F$ nuclei in $V_R$. Note that in all of the above formulas the various intensities (i.e., $I_R$, $I_c^{avg}$, $I_c^{(i)}$) can be normalized to the image noise, and thus the above formulas can be equivalently expressed in terms of the appropriate SNR values for the particular regions. Thus, there are many ways to estimate the number of labeled cells, $N_c$, and many similar forms of these basic expressions can be derived by basic mathematical manipulations, however, all rely on the same basic content contained within the input parameters described by (i-x). Furthermore, quantification of labeled cells in an ROI need not be expressed in terms of absolute numbers or effective cell numbers. Other quantitative indices can be derived that are indicative of the amount of cells in an ROI. For example, one can calculate the ratio $I_c^{avg}/I_R$, or the ratio of the average SNR values observed in the ROI and the reference; all of these fall within subsets of the above expressions and/or the parameters.

It is noted that the above analysis of cell numbers and related indices assume that the $^{19}F$ NMR relaxation times (i.e., particularly T1 and/or T2) of the fluorocarbon label is approximately the same as material in the calibrated $^{19}F$ reference standard. In the case that the relaxation times are not comparable, one of skill in the art can readily correct for this by employing the known MRI intensity equations of the particular imaging protocol being used, expressed in terms of T1 and T2.

Optionally, the $^{19}F$ MRI data set of the subject material can undergo post-processing before the actual cell quantification calculation is performed (as described above). For example, post-processing algorithms may include "de-noising" the $^{19}F$ data set. This can be accomplished by, for example, by thresholding the image to cut off low-intensity noise; this involves rescaling the image intensity so that low values are set to zero. In magnitude MRI images, random Johnson noise is often apparent and uniformly

distributed across the image FOV. It is well know in the art that one can threshold out the low–level image intensity so that regions known to contain no true signal (i.e. devoid of $^{19}F$ and/or $^{1}H$ nuclei ) appear to have a null or very near-null intensity. This process can be performed in an ad-hoc fashion (i.e. "manually" or by visual inspection), or by using a computer algorithm. In other embodiments, de-noising of the data set can be achieved by using other algorithms, for example using wavelet analysis, and many methods are known in the art for image de-noising. The following references are incorporated in their entirety herein: Khare, A., et al., INTERNATIONAL JOURNAL OF WAVELETS MULTIRESOLUTION AND INFORMATION PROCESSING, 3 (4): 477-496 DEC 2005; Cruz-Enriquez, H., et al., IMAGE ANALYSIS AND RECOGNITION, 3656: 247-254 2005; Awate, SP., et al., INFORMATION PROCESSING IN MEDICAL IMAGING, PROCEEDINGS, 3565: 677-688 2005; Ganesan, R., et al., IIE TRANSACTIONS, 36 (9): 787-806 SEP 2004; Scheunders, P., IEEE TRANSACTIONS ON IMAGE PROCESSING, 13 (4): 475-483 APR 2004; Ghugre, NR., MAGNETIC RESONANCE IMAGING, 21 (8): 913-921 OCT 2003; Bao, P., et al., IEEE TRANSACTIONS ON MEDICAL IMAGING, 22 (9): 1089-1099 SEP 2003; Wu, ZQ., et al., ELECTRONICS LETTERS, 39 (7): 603-605 APR 3 2003; LaConte, SM., et al., MAGNETIC RESONANCE IN MEDICINE, 44 (5): 746-757 NOV 2000; Laine, AF., ANNUAL REVIEW OF BIOMEDICAL ENGINEERING, 2: 511-550 2000; Zaroubi, S., et al., MAGNETIC RESONANCE IMAGING, 18 (1): 59-68 JAN 2000; Nowak, RD., IEEE TRANSACTIONS ON IMAGE PROCESSING, 8 (10): 1408-1419 OCT 1999; and Healy, DM., et al., ANNALS OF BIOMEDICAL ENGINEERING, 23 (5): 637-665 SEP-OCT 1995.

Other types of post-processing algorithms are know in the art that can be applied to the $^{19}F$ MRI data set before or after quantification, such as zero-filling (A Handbook of Nuclear Magnetic Resonance, 2$^{nd}$ Edition, Ray Freeman, Addison Wesley Longman Press 1997) and various image interpolation, de-noising, and image smoothing algorithms (for example, see The Image Processing Handbook, 3$^{rd}$ Edition, John C. Russ, CRC Press/IEEE Press).

In certain embodiments the above set of key parameters (i-x) can be used to derive quantitative or statistical measures of the accuracy or confidence of the measured number of labeled cells or related indices. $^{19}F$ MRI/MRS data sets are often subject to SNR

limitations within ROI, and thus it is often useful to calculate a metric of the confidence or accuracy of the measurement. Many methods are known in the art for the statistical analysis of MRI and other biomedical-type images. The claimed embodiment is understood to encompass these known methods.

5. Computer methods

Methods for quantifying labeled cells will typically be conducted with the aid of a computer, which may operate software designed for the purpose of such quantification. Such software may be a stand-alone program or it may be incorporated into other software, such as MRI image processing software. Figure 53 shows a functional block diagram of general purpose computer system 200 for performing the functions of the computer according to an illustrative embodiment of the disclosure. The exemplary computer system 200 includes a central processing unit (CPU) 202, a memory 204, and an interconnect bus 206. The CPU 202 may include a single microprocessor or a plurality of microprocessors for configuring computer system 200 as a multi-processor system. The memory 204 illustratively includes a main memory and a read only memory. The computer 200 also includes the mass storage device 208 having, for example, various disk drives, tape drives, etc. The main memory 204 also includes dynamic random access memory (DRAM) and high-speed cache memory. In operation, the main memory 204 stores at least portions of instructions and data for execution by the CPU 202.

The mass storage 208 may include one or more magnetic disk or tape drives or optical disk drives, for storing data and instructions for use by the CPU 202. At least one component of the mass storage system 208, preferably in the form of a disk drive or tape drive, stores the database used for processing the cell quantification of the disclosure. The mass storage system 208 may also include one or more drives for various portable media, such as a floppy disk, a compact disc read only memory (CD-ROM), or an integrated circuit non-volatile memory adapter (i.e. PC-MCIA adapter) to input and output data and code to and from the computer system 200.

The computer system 200 may also include one or more input/output interfaces for communications, shown by way of example, as interface 210 for data communications via the network 212. The data interface 210 may be a modem, an Ethernet card or any other suitable data communications device. To provide the functions of a computer, the data

interface 210 may provide a relatively high-speed link to a network 212, such as an intranet, internet, or the Internet, either directly or through another external interface. The communication link to the network 212 may be, for example, optical, wired, or wireless (e.g., via satellite or cellular network). Alternatively, the computer system 200 may include a mainframe or other type of host computer system capable of Web-based communications via the network 212.

The computer system 200 also includes suitable input/output ports or uses the interconnect bus 206 for interconnection with a local display 216 and keyboard 214 or the like serving as a local user interface for programming and/or data retrieval purposes. Alternatively, server operations personnel may interact with the system 200 for controlling and/or programming the system from remote terminal devices via the network 212.

The computer system 200 may run a variety of application programs and stores associated data in a database of mass storage system 208. One or more such applications may enable the receipt and delivery of messages to enable operation as a server, for implementing server functions relating to quantification.

The components contained in the computer system 200 are those typically found in general purpose computer systems used as servers, workstations, personal computers, network terminals, and the like. In fact, these components are intended to represent a broad category of such computer components that are well known in the art. Certain aspects of the disclosure may relate to the software elements, such as the executable code and database for the server functions of the quantification system.

The disclosure will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present application, and are not intended to limit the disclosure.

## EXAMPLES

Data presented in WO2005072780 demonstrated that immune cells and other cell types cells could readily be labeled with a fluorocarbon imaging reagent *ex vivo*, and that labeled cells could be detected *in vivo*. Data presented in US provisional application No. 60/792003 demonstrated the further feasibility of the disclosed methods is presented, including data demonstrating the *in vivo* quantification of labeled cells. The exemplary

embodiment of the disclosure develops novel imaging reagents and emulsions and evaluates their efficacy in tissue culture.

## 1. Imaging Reagent Preparation

Simple, very efficient and scalable synthetic methods are presented for PFPE derivatization and nanoemulsion preparations. PFPE ester 39 proved to be a versatile starting material for synthesis of a variety of nanoparticles, including dual fluorescent-19F MRI reagents, self deliverable PFPE nanoparticles and nanoparticles with highly efficient uptake in both phagocytic and non-phagocytic cell types. Emulsions that promote cellular uptake and emulsions that would promote serum stability are presented. The emulsions produced were highly stable at storage temperatures (4 and 25 °C) and body temperature (37 °C). Simple changes in the emulsification process and simple chemical modifications of PFPE end groups allow fine tuning of nanoparticle properties towards specific applications.

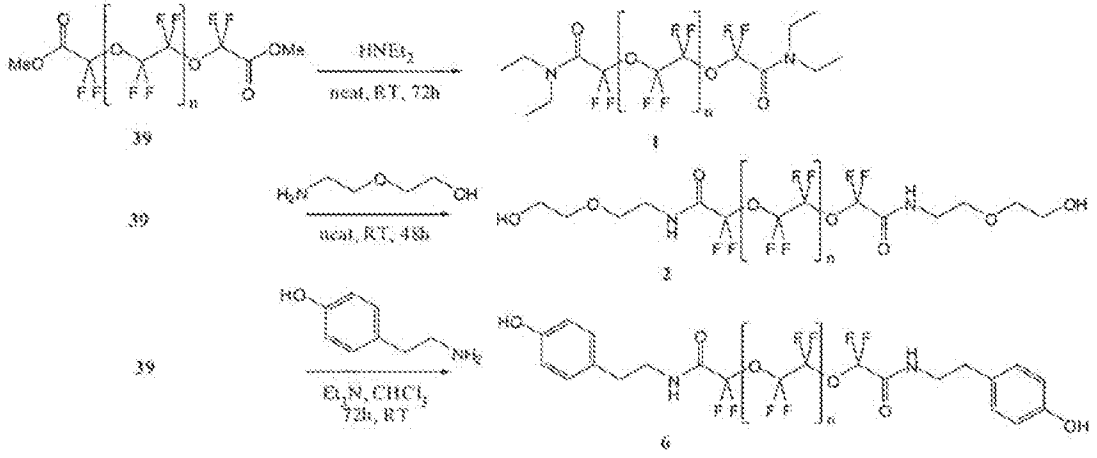
Examples of chemical modifications of PFPE end groups to variety of amides that can serve as both emulsion stabilizers or conjugation sites are presented.

Synthesis of symmetrical PFPE diamides:

In general, PFPE methyl ester oil (39) was mixed with excess amine, either neat liquid or a solution of the amine in an appropriate solvent, as specified in Scheme 1, under inert atmosphere (argon or nitrogen) at room temperature. The reaction was allowed to stir 1-3 days, depending on the amine reactivity, to allow complete conversion. When amine was added as a solid, trifluorotoluene or trifluoroethanol was used as solvent and the temperature was increased to 40, 50, 60 or 70 °C depending on the amine reactivity (Scheme 2). Reaction progress was followed by 1H NMR monitoring the disappearance of the methyl ester peak (Figure 45). After reaction completion, high vacuum was employed to remove resulting methanol and the excess unreacted amine. When non-volatile amines were used, an acidic aqueous wash was used to extract excess amine, and the desired PFPE amide was extracted with appropriate solvent. A recent patented procedure (US 7,038,068 B2) describing purification of linear PFPEs by selective organic

solvent extractions, was used for PFPE amide derivative purification. When organic solvent extraction was not enough, adsorption to fluorous phase silica gel (FluoroFlash, Fluorous Inc.) and elution of the purified product with methanol followed by trifluoroethanol or perfluorohexanes was used. Structures of the PFPE amide derivatives were confirmed by 1H, 13C, 19F NMR and MALDI-TOF mass spectrometry. Compounds 1, 2, and 6 were prepared according to this general procedure, as is shown in Scheme 1, wherein n, independently for each occurrence, represents an integer from 4 to 16.

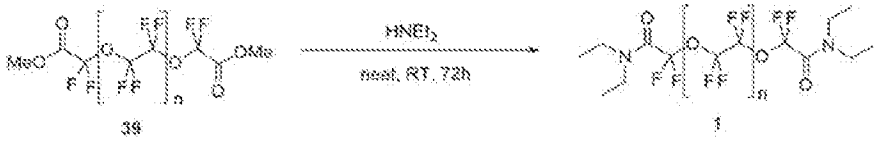
Scheme 1:




**General Experimental Procedures**

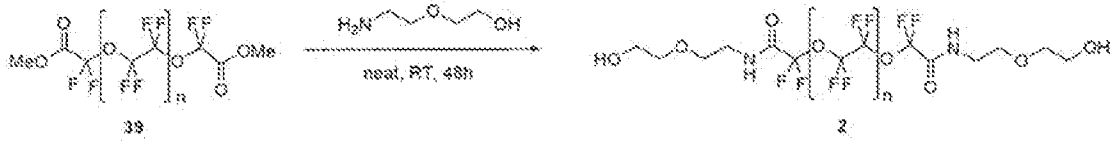
All reactions were performed in flame dried round bottom flasks under a nitrogen or argon atmosphere unless indicated otherwise. All reagents and solvents were purchased from Aldrich, Acros, TCI or Lancaster Synthesis and used without further purification. THF and DMF were purchased anhydrous grade and used without further purification. 1H and 13C NMR spectra were obtained on a Bruker Avance 300 at 300 and 75 MHz in $CDCl_3$ unless otherwise noted. Chemical shifts were reported in parts per million (ppm) using the residual solvent signal as an internal standard. $^1H$ NMR spectra are tabulated as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, b = broad), number of protons and coupling constant(s). $^{13}C$ NMR spectra were acquired using a proton decoupled pulse sequence with a pulse sequence delay of 5 sec. $^{19}F$ NMR was obtained on Bruker Avance 500 at 470 MHz in water or acetone. Chemical shifts were reported as parts per million (ppm) using Trifluoroacetic acid as an

internal standard with $^{19}F$ chemical shift at -76.0 ppm. Low resolution and high resolution mass spectra (MS and HRMS, respectively) were obtained in positive ion mode by matrix-assisted laser desorption ionization (MALDI) using a pentafluorobenzoic acid matrix (Marie, A.; Alves, S.; Fournier, F.; Tabet, J.C. Analytical Chemistry (2003), 75, 1294-1299) and an Applied Biosystems 4700 MALDI-TOF/TOF-MS.



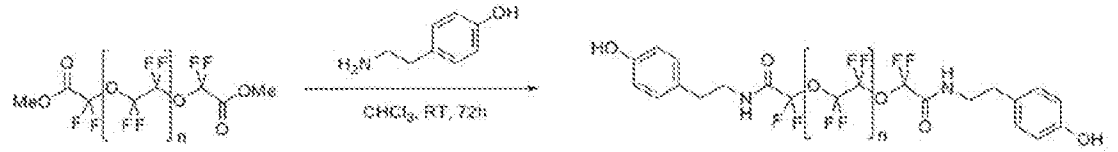

39 1

**Perfluoropolyether diethyl amide (1). General Procedure A, PFPE coupling to amines with low boiling point:** A literature procedure was used with extensive modifications (Piacenti, F.; Camaiti, M. Journal of Fluorine Chemistry (1994), 68, 227-235). Perfluoropolyether methyl ester **39** (81.0 g, 50 mL, 46.3 mmol, Ave MW = 1750) and diethylamine (81.3 g, 115 mL, 1.1 mol), were mixed in a round bottom flask (500 mL) under an argon atmosphere, and stirred at room temperature for 72 h. The excess amine and methanol formed during the reaction were removed by vacuum. The resulting brown oil was washed with THF (200 mL) and the oil and THF phase separated. The lower phase was collected and the residual solvent was removed by vacuum which afforded purified perfluoropolyether diethylamide **1** (73.1 g, 45 mL, 39.5 mmol, 85 %) as a yellow to orange clear oil: $^1H$ NMR (300MHz, $d_6$-acetone) δ $CH_2$ 3.57 – 3.42 (4H, m), $CH_2$ 2.85 – 2.75 (4H, m), $CH_3$ 1.27 – 1.90 (6H, m), $CH_3$ 1.22 – 1.1 (6H, m); $^{13}C$ NMR (75MHz, $d_3$-trifluoroethanol) δ C=O 159.2 (t, $J_{C-F}$ = 32Hz), $OCF_2$ 120.2 (t, $J_{C-F}$ = 48Hz), $OCF_2CF_2$ 116.3 (t, $J_{C-F}$ = 44Hz), $CF_2O$ 112.5 (t, $J_{C-F}$ = 48Hz), $CH_2$ 44.1, $CH_2$ 43.8, $CH_3$ 14.0, $CH_3$ 12.1; $^{19}F$ (500MHz, referenced TFA -76.0) $OCF_3$ -58.4, $OCF_2NEt_2$ -74.4, $(CF_2CF_2O)$ -91.1; MS (MALDI-TOF, positive ion) $(CF_2CF_2O)_{16}$ 2194.9022 $[M+Na]^+$, $(CF_2CF_2O)_{15}$ 2078.8904 $[M+Na]^+$, $(CF_2CF_2O)_{14}$ 1962.9107 $[M+Na]^+$, $(CF_2CF_2O)_{13}$ 1846.9364 $[M+Na]^+$, $(CF_2CF_2O)_{12}$ 1730.9573 $[M+Na]^+$, $(CF_2CF_2O)_{11}$ 1614.9851 $[M+Na]^+$, $(CF_2CF_2O)_{10}$ 1499.0309 $[M+Na]^+$, $(CF_2CF_2O)_9$ 1499.0309 $[M+Na]^+$, $(CF_2CF_2O)_8$ 1383.0537 $[M+Na]^+$, $(CF_2CF_2O)_7$ 1267.0799 $[M+Na]^+$, $(CF_2CF_2O)_6$ 1151.1030 $[M+Na]^+$, $(CF_2CF_2O)_5$ 1035.1268 $[M+Na]^+$. Only the main monomer repeats are shown. Mass spectrometry data shows a distribution of PFPE linear molecules with different lengths, (Figure 50).




39 2

**Perfluoropolyether O-(2-Hydroxyethyl)ethanolamide (2).** General procedure A was used with some modifications. 2-(2-Aminoethoxy) ethanol (2.2 g, 20.9 mmol) was mixed with triethylamine (4.2 mL, 30 mmol) at room temperature in a round bottom flask (100 mL) under an argon atmosphere. Perfluoropolyether methyl ester **39** (17.5 g, 10.8 mL, 10 mmol, Ave MW = 1750) was added. The reaction mixture turns brown and clear immediately while its temperature rises to 40°C. The reaction mixture is allowed to cool to room temperature and stirring continued for 48 h. The excess triethylamine and methanol, formed during the reaction, were removed by vacuum. The crude reaction product was loaded on the Fluoro Flash short column (20 g) and washed with 50mL of acetonitrile. Elution with methanol (100 mL) and concentration in vacuo afforded perfluoropolyether O-(2-Hydroxyethyl)ethanolamide **2** (11.1 g, 5.9 mmol, 59 %) with average MW 1895, as a dark yellow oil. $^1$H NMR (300 MHz, $d_6$-acetone) δ NH 8.55 (2H, singlet), $OCH_2$ 3.62 (8H, t, $J_{H-H}$ = 5.1 Hz), $CH_2N$ 3.53 (8H, t, $J_{H-H}$ = 4.8 Hz), OH 3.30 (2H, singlet); $^{19}$F (470 MHz, referenced TFA -76.0) $OCF_2CONHCH_2$ -78.8, $(CF_2CF_2O)$ -89.5.




39 6

**Perfluoroplyether tyramide (6). General Procedure B, PFPE coupling to amines as solids and high boiling point liquids.** 4-(2-Aminoethyl) phenol (0.8 g, 6.0 mmol) was dissolved in chloroform (25 mL) in a round bottom flask (100 mL) and triethylamine (0.9 g, 1.2 mL, 8.6 mmol) was added under an argon atmosphere at room temperature while stirring. Perfluoropolyether methyl ester **39** (5.0 g, 3.1 mL, 2.9 mmol, Ave MW = 1750) was added at once and the reaction mixture was stirred at room temperature for 72 h. The excess triethylamine and methanol formed during the reaction were removed by vacuum. Previously reported fluorous solid phase extraction method was followed with some

modifications (Zhang, W.; Curran, D. Tetrahedron (2006), 62, 11837–11865). Fluorous silica gel adsorbs perfluoropolyether and allows easy elution of all non fluorinated organic compounds. The purification procedure was as follows. The crude reaction product was dissolved in acetone and loaded onto Fluoro Flash™ short column (10g) packed wet with acetonitrile/water (95:5 v/v). The crude PFPE oil in acetone was loaded by gravity and then the Fluoro Flash column washed with acetonitrile/water (8:2 v/v) mixture (50 mL) followed by methanol (200 mL) and methanol/trifluoroethanol (1:1 v/v) (100 mL). Fluorophilic methanol fractions were collected, and the residual solvent was removed by vacuum which afforded purified perfluoropolyether tyramide 6 (4.8 g, 2.5 mmol, 86.2 %) with average MW 1950 as a yellowish waxy solid: $^1$H NMR (300MHz, $d_6$-acetone) δ CH ($C_6H_6$) 7.02 (4H, d, $J_{H-H}$ = 4.2 Hz), CH ($C_6H_6$) 6.75 (4H, d, $J_{H-H}$ = 4.2 Hz), $CH_2$ 3.48 (4H, t, $J_{H-H}$ = 7.8 Hz), $CH_2$ 2.76 (4H, t, $J_{H-H}$ = 7.5 Hz); $^{19}$F (470 MHz, referenced TFA -76.0, $d_6$-acetone) $OCF_3$ -56.4, $OCF_2CONCH_2$ -78.9, ($CF_2CF_2O$) -89.4.

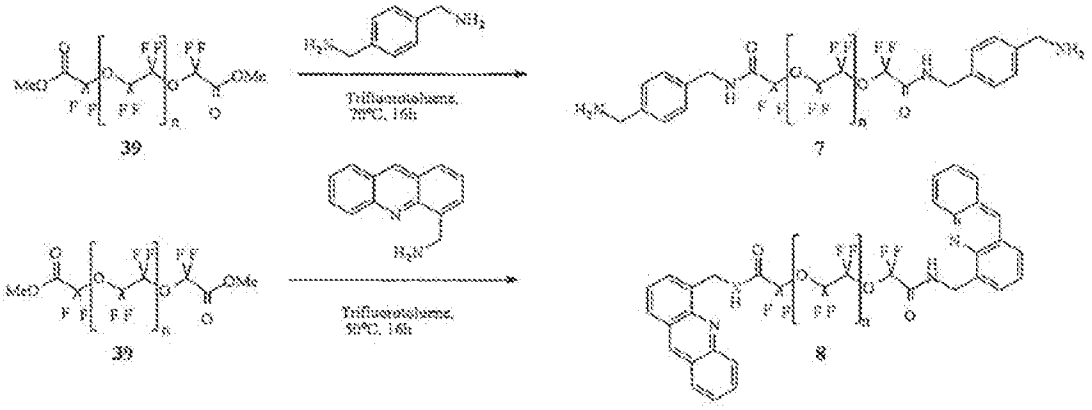
**$^{19}$F NMR Analysis**

The $^{19}$F NMR spectra can be used to unambiguously distinguish PFPE ester from PFPE amides. The $CF_2COOMe$ preterminal in PFPE ester (39) has a chemical shift of -80.3 ppm (Figure 49). In the PFPE-diethyl amide (1), the $CF_2CON$ preterminal has chemical shift of -74.4 ppm (Figure 48), and in the PFPE-tyramide conjugate where the end group has a secondary amide, the $CF_2CON$ preterminal has chemical shift of -77.8 ppm (Figure 52).

In the case of the BODIPy-TR PFPE conjugate (compounds 16 and 17), the most diagnostic peak was the $^{19}$F on the dye at -127.7 ppm for $CF_3$. These results indicate the usefulness of $^{19}$F NMR in the analysis of PFPE modifications, PFPE emulsion products, and the PFPE uptake evaluation in target cells.

Similarly, compounds 7 and 8 were prepared according to the reactions in Scheme 2, wherein n, independently for each occurrence, represents an integer from 4 to 16.

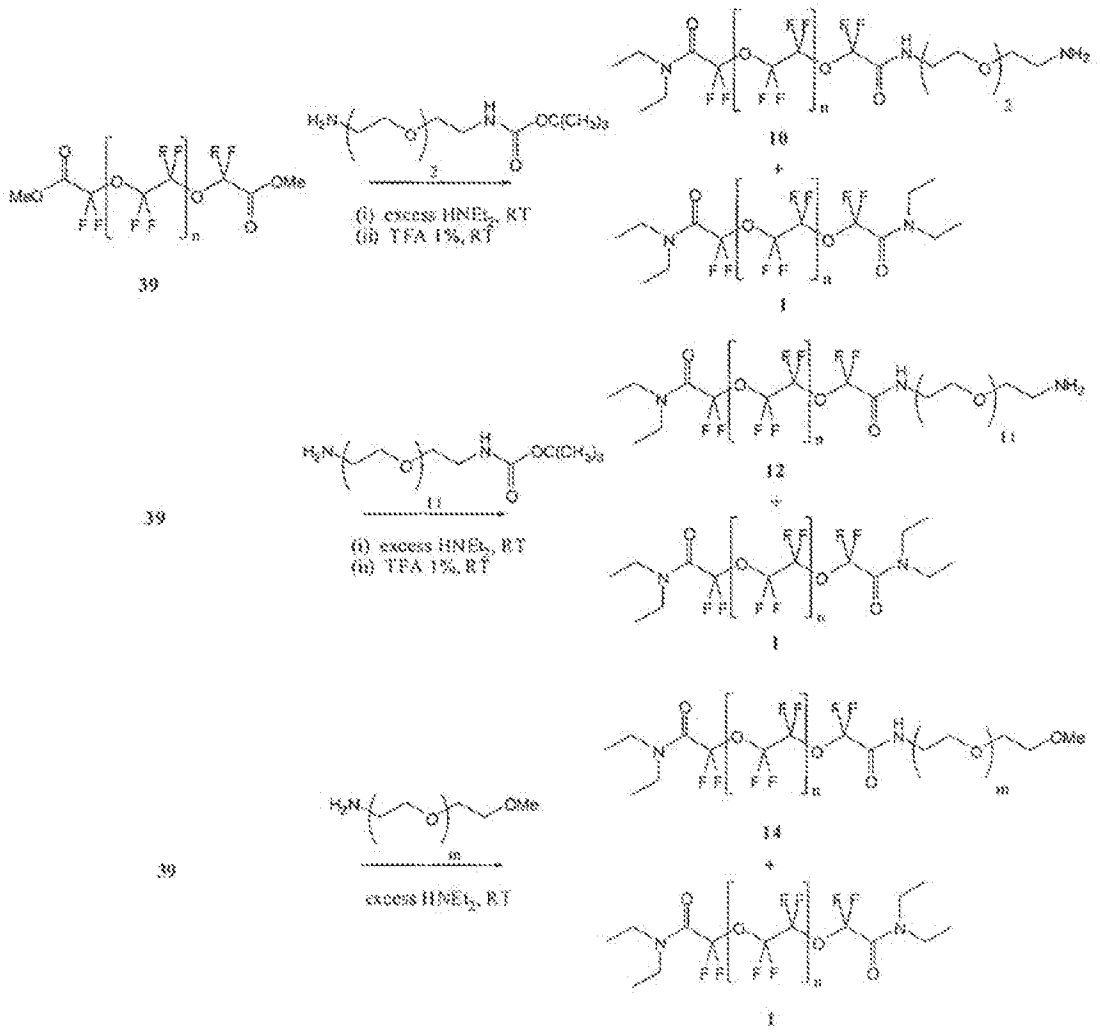
Scheme 2:



Synthesis of Compositions:

Compositions comprising a compound of formula 1 and a compound of formula 10; a compound of formula 1 and a compound of formula 12; and a compound of formula 1 and a compound of formula 14 were prepared according to the reactions shown in Scheme 3, wherein n, independently for each occurrence, represents an integer from 4 to 16. In general, PFPE methyl ester (39) was first reacted with a primary amine of a PEG moiety and the coupling was allowed sufficient time to complete, usually 1-3 days at room temperature. The rest of free, unmodified ester end groups were converted to diethyl amide in the final step, and after vacuum removal of the excess diethyl amine and methanol, the end product contained defined molar ratios of PEG coupled to PFPE blended with PFPE diethyl amide (1). The ratio of two different modifications of PFPE end groups were easily confirmed by 1H and 13C NMR. Any uncoupled primary amine was removed by selective organic solvent extraction, usually ethanol or THF. The composition comprising a compound of formula 10 and a compound of formula 1 was produced in a molar ratio of 1:2. The composition comprising a compound of formula 12 and a compound of formula 1 was produced in a molar ratio of 1:4. The composition comprising a compound of formula 14 and a compound of formula 1 was produced in a molar ratio of 1:10.

Scheme 3:



Compositions comprising a compound of formula 1 and a compound of formula 16; and a compound of formula 1 and a compound of formula 18 were prepared according to the reactions shown in Scheme 4, wherein n, independently for each occurrence, represents an integer from 4 to 16.

**Fluorescent "blended" PFPE amides (FBPAs).**

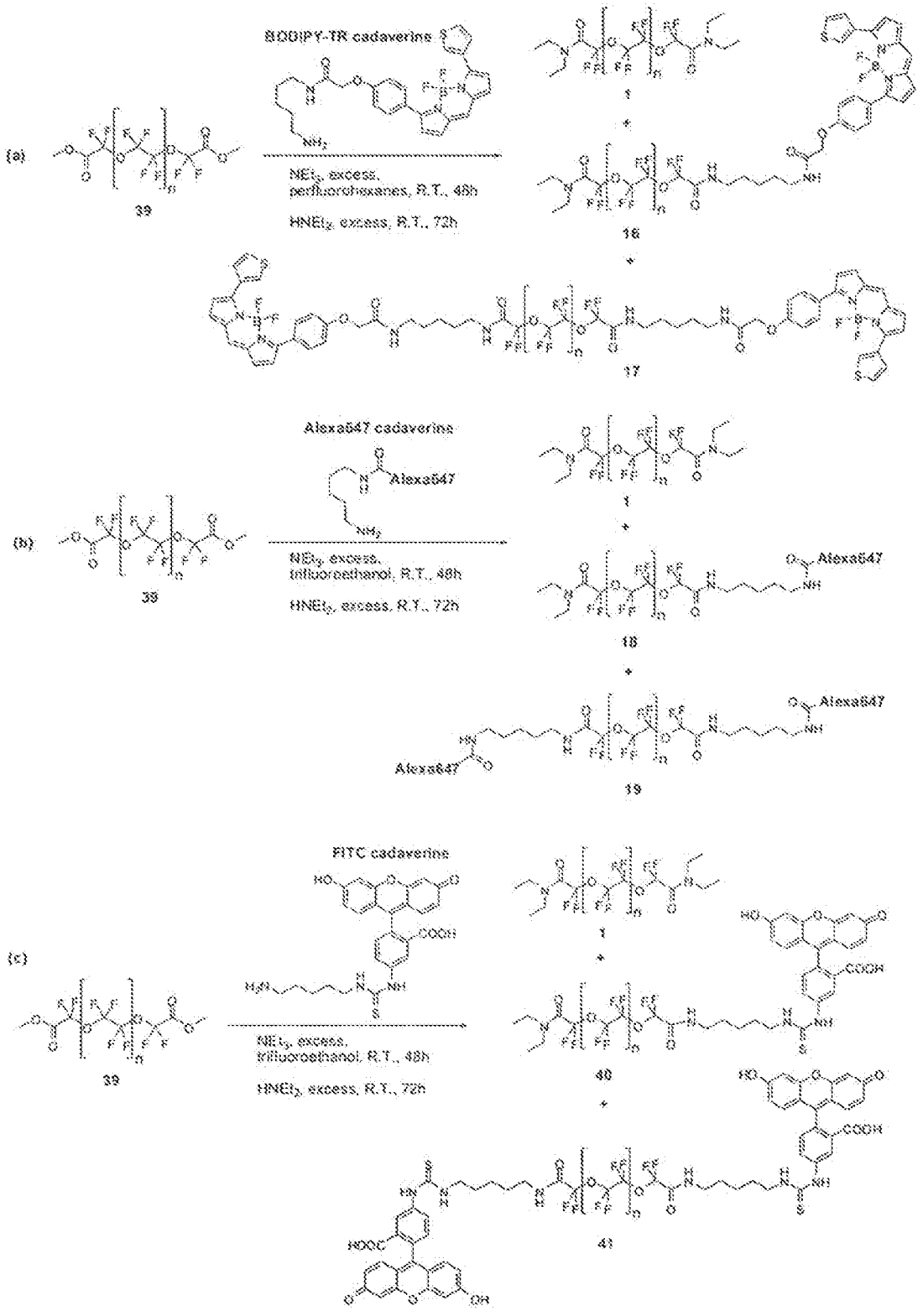
**General procedure.** The fluorescent dyes (FITC, BODIPy-TR and Alexa647) were purchased from Molecular Probes, Eugene, OR as mono-conjugates to 1,5-diaminopentane (cadaverine), where the unmodified primary amine allows direct coupling to PFPE ester 39. Our synthetic approach for fluorescent PFPEs relies on the highly efficient initial coupling of the primary amine of the fluorescent dye conjugate to PFPE ester 39, as described in the model reaction above, Scheme 3, wherein complete conversion (>99 %) is

observed by $^1$H NMR after 48 h at rt. The final product is FBPA, a perfectly blended mixture of PFPE derivatives comprised of dye di-conjugate (e.g., compound 17, 19, or 41), dye mono-conjugate (e.g., compound 16, 18, or 40) and PFPE amide 1 (Scheme 4).

Briefly, fluorescent dye (FITC, BODIPy-TR or Alexa647) cadaverine conjugate was added at 0.5 to 2.0 mol % to a solution of PFPE ester 1 in trifluoroethanol or perfluorohexanes. The reaction was allowed to proceed at room temperature under inert atmosphere for up to 48 h. The remaining free, unmodified PFPE ester 39 end groups were converted to diethyl amide in the final step, and the excess unreacted diethyl amine and the side product methanol were removed by vacuum. Since the fluorescent dye was conjugated directly to only a portion of PFPE end groups thereby forming a secondary amide, while the remainder of PFPE end groups were capped with tertiary amide, fluorescence and UV/VIS absorbance measurements of labeled PFPE were used for coupling efficiency estimates. Any uncoupled primary amine was removed by selective organic solvent extraction, usually ethanol, or eluted by fluorophobic solvent (e.g., acetonitrile/water, 4:1) from the fluorous phase silica gel.

PFPE ester 39 is fully soluble only in trifluoroethanol, trifluorotoluene and perfluorohexanes. Trifluoroethanol was the only solvent that solubilizes both the dye and the PFPE oil. BODIPy-TR cadaverine was added at low concentration (5 mg dye/1 mL of PFPE oil) and allowed to react for 48 h at r.t. in trifluoroethanol or perfluorohexanes.

Scheme 4


BODIPY-TR cadaverine
(a)
39
NEt3, excess,
perfluorohexanes, R.T., 48h
HNEt2, excess, R.T., 72h
1
16
17
Alexa647 cadaverine
(b)
39
NEt3, excess,
trifluoroethanol, R.T., 48h
HNEt2, excess, R.T., 72h
1
18
19
FITC cadaverine
(c)
39
NEt3, excess,
trifluoroethanol, R.T., 48h
HNEt2, excess, R.T., 72h
1
40
41

**BODIPy-TR PFPE amide (FBPA composed of PFPE amide 1, 16 and 17).** A flame dried round bottom flask (50 mL) was charged with perfluoropolyether methyl ester 39 (3.96 mL, 3.67 mmol) under an argon atmosphere. BODIPy-TR cadaverine (0.02 g, 0.04 mmol) was added to PFPE oil as a solid and, after extensive stirring, perfluorohexane solvent was added (3.00 mL) followed by triethylamine (1.50 mL, 10.8 mmol). (Alternatively, trifluoroethanol can be used as a solvent for the fluorescent conjugate such that the BODIPy-TR Cadaverine is added as solution in trifluoroethanol. This approach is used on large scale.) The reaction mixture was allowed to stir at r.t. for 48 h protected from light. Diethylamine (9.50 mL, 91.8 mmol) was added to convert all unreacted ester end groups into tertiary amide, and the reaction continued for 72 h at r.t. The excess diethyl amine and methanol were removed by vacuum, and the final dark blue oil was further purified as follows. The product was subjected to ethanol wash (10 mL), and then purified by fluorous solid phase filtration. A FluoroFlash column was packed wet in acetonitrile/water (95:5 v/v). Product was loaded on the column using negative pressure. The column was washed with acetonitrile (100 mL), and the fluorophobic fractions combined, concentrated to dryness in vacuo and redissolved in trifluoroethanol (20 mL). The column was then washed with THF (100 mL) followed by trifluoroethanol (50 mL), and the fluorophilic fractions combined, concentrated to dryness and the residue dissolved in trifluoroethanol (20 mL). These solutions were used for spectrophotometric measurements. BODIPy-TR cadaverine was dissolved in trifluoroethanol, and a standard curve was constructed by measuring absorbance at 593 nm. The BODIPy-TR concentrations in the fluorophilic and fluorophobic fractions were estimated from the standard curve. BODIPy-TR coupling yield was 46.8%, and the final concentration of the fluorescent dye in neat oil was 4.6 mM. The FBPA oil was stored in the dark at r.t. until used for nanoemulsion preparations. The absorbance standard curve is shown in Figure 59.

Due to low concentration of the dye in FBPA, $^{1}H$ and $^{13}C$ NMR analysis did not produce useful data. The fluorous phase extractions were performed to remove any uncoupled organic dye from the highly fluorinated PFPE oil at the final step of the FBPA preparations. Fluorous phase solid extractions and UV spectrophotometry were used to confirm the conjugation and to calculate coupling yield.